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Cell Biology and Molecular Basis of Denitrification†

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† Dedicated to the memory of Jacobo Cárdenas SJ.

INTRODUCTION

Denitrification constitutes one of the main branches of the global nitrogen cycle sustained by bacteria. The cycle manifests the redox chemistry inherent to the principal natural inorganic nitrogen species dinitrogen, ammonia, and nitrate, as shown in its essential, minimized version in Fig. 1. Nitrogen is introduced into the biosphere by biological and chemical fixation of dinitrogen (N_2) and removed from there again by denitrification. In doing this, denitrification catalyzes successively N—N bond formation in the transformation of its intermediates nitric oxide (NO) and nitrous oxide (N_2O) to the next-lower oxidation state. The bacterial process is nearly exclusively a facultative trait. Its expression is triggered in the cell by the environmental parameters low oxygen tension and availability of an N oxide.

Denitrification is part of the bioenergetic apparatus of the bacterial cell, where the N oxyanions nitrate and nitrite and the gaseous N oxides NO and N_2O serve in lieu of dioxygen (O_2) as terminal acceptors for electron transport phosphorylation. It is clearly the role of denitrification in the global N cycle and in cellular bioenergetics that makes a detailed knowledge of this process essential.

More recent concerns related to denitrification begin to foster research in this area. Nitrate, irrespective of its role as essential plant nutrient, has become a pollutant of groundwater and surface water, causing a major problem for the supply of drinking water. N_2O is next to CO_2 and CH_4 in its importance as a potent greenhouse gas (its efficiency is much higher than that of CO_2), and, together with NO, it is of much concern in terms of the ozone chemistry of the atmosphere (167, 204). Wastewater treatment plants may contribute to the emission of N_2O and increase the greenhouse effect (624). The residence time of N_2O in the atmosphere is estimated to be 150 years (453). Over the past decades, a constant increase has been observed; fertilizer denitrification is thought to contribute significantly to this increase. Biomass burning and nylon manufacture are among the more recently discovered significant anthropogenic N_2O sources (152, 812). The flux of gases between the soil and the atmosphere due to bacterial activities has been described in detail, with specific attention being paid to NO and N_2O (154).

The distribution of denitrification among the prokaryotes does not follow a distinct pattern. The reaction is carried out by a diversity of bacteria belonging taxonomically to the various subclasses of the *Proteobacteria*. Denitrification also extends beyond the bacteria to the archaea, where it is found among the halophilic and hyperthermophilic branches of this kingdom and may have evolutionary significance. Intriguingly, the NO- and N_2O-utilizing enzymes share structural elements with certain terminal oxidases of the aerobic respiratory chain. An entirely new development is the recognition of the core enzymes of denitrification in the mitochondria of certain fungi (460).

Emphasis will be given here to the denitrification process in the strict sense. Nitrite reductase is the key enzyme of denitrification in catalyzing the first committed step that leads to a gaseous intermediate. For the denitrification process to lead to dinitrogen formation, the nitrite reductase reaction is complemented by the activity of two distinct metalloenzymes, which

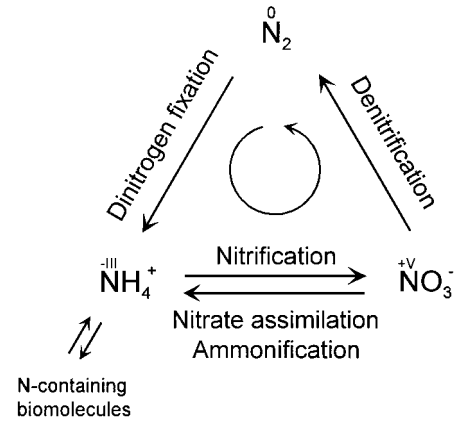

FIG. 1. Biogeochemical nitrogen cycle sustained by prokaryotes. The roman numerals give the formal oxidation state of the principal nitrogen species of the cycle. Dinitrogen fixation depends on the nitrogenase reaction, which proceeds without free intermediates. Denitrification comprises four enzymatic steps, generating the intermediates nitrite, nitric oxide, and nitrous oxide. The only oxidative segment of the cycle is nitrification, which proceeds from ammonia via hydroxylamine (NH_2OH) to nitrite, catalyzed by members of the genus *Nitrosomonas*. Nitrite is further oxidized to nitrate by *Nitrobacter* spp. Nitrate assimilation and ammonification each involve nitrite as the single free intermediate. The incorporation of ammonia into nitrogen-containing biomolecules usually starts with the glutamine synthetase reaction.

TABLE 1. Dissimilatory and assimilatory branches of nitrate reduction in the prokaryotic N cycle

Dissimilatory branch		Assimilatory branch
Denitrification (energy conservation, electron sink)	Ammonification (electron sink, detoxification, energy conservation)	Assimilation (biosynthesis of nitrogen-containing compounds)
Respiratory nitrate reduction $NO_3^- \rightarrow NO_2^-$ (nitrite excreted or reduced further)		Assimilatory nitrate reduction $NO_3^- \rightarrow NO_2^-$ (nitrite reduced further)
↓	↓	↓
Denitrification sensu stricto, nitrite and nitric oxide respiration $NO_2^- \rightarrow NO \rightarrow N_2O$ (gases may be set free)	Ammonifying nitrite reduction $NO_2^- \rightarrow NH_4^+$ (ammonia excreted)	Assimilatory nitrite reduction $NO_2^- \rightarrow NH_4^+$ (ammonia incorporated)
↓	↑	↑
Nitrous oxide respiration $N_2O \rightarrow N_2$	Denitrification sensu lato associated with both branches $NO_3^-/NO_2^- \rightarrow N_2O$	

use NO or N_2O as substrates. Because much work on denitrification has been done with pseudomonads and several strains of *Paracoccus*, members of these groups will be prominent throughout this article, but most findings from other denitrifiers are included to present the entire scope and the manifold cell biological ramifications of the field.

The process embraces important biological problems of structure and function in microbial bioenergetics and requires highly interdisciplinary approaches for its study. This review centers on the interlacing of denitrification with other cellular processes, emphasizing the concepts emerging from the substantial advances in the biochemistry and genetics of this process during the past decade. Denitrification will be covered in terms of the structural and functional aspects of the participating enzymes and with a focus on general cellular aspects to include the genetic basis and regulation of the process, the biological chemistry of activation and transformation of small inorganic molecules, transport and assembly processes related to metalloprotein biogenesis, cofactor biosynthesis, and anaerobic gene expression and gene activation by environmental factors. Denitrification has been covered twice in this journal in the past (459, 642); in addition, a monograph has described the earlier developments in this area (643). Bioenergetic (793, 794) and environmental (678, 820) aspects of the process have also been emphasized in reviews elsewhere.

The following abbreviations are used in this article: ABC, ATP-binding cassette; ALA, 5-aminolevulinic acid; asc, ascorbate; BV, benzyl viologen; cAMP; cyclic AMP; COX, cytochrome-*c* oxidase; CRP, cyclic AMP receptor protein; CuNIR, Cu-containing respiratory nitrite reductase; DDC, diethyl dithiocarbamate; DMSO, dimethyl sulfoxide; EXAFS, extended X-ray absorption fine structure; EPR, electron paramagnetic resonance; IHF, integration host factor; MCD, magnetic circular dichroism; MGD, molybdopterin guanine dinucleotide; MPT, molybdopterin; MV, methyl viologen; ORF, open reading frame; PMS, phenazine methosulfate; SDS, sodium dodecyl sulfate; SOD, superoxide dismutase.

TERMINOLOGY

Denitrification originally described a phenomenon, i.e., the loss of fixed nitrogen from the viewpoint of the N balance of a fermenting microbial community. The term was preserved by Kluyver when he formulated his unifying concept of cellular bioenergetics and recognized that denitrification allows bacteria a respiratory way of anaerobic life (458). An N oxide, instead of oxygen, serves as the electron acceptor for the generation of an electrochemical gradient across the cytoplasmic membrane. Because a bacterial cell often disposes electrons over several terminal oxidoreductases that use different N oxides, their concerted action results in the sequential transformation of nitrate to N_2. Denitrification is also found in chemolithotrophic organisms. The oxidation of an inorganic source of reductant coupled to N oxide reduction is sometimes termed autotrophic denitrification.

The phenomenological definition of denitrification is thus the dissimilatory transformation of nitrate or nitrite to a gas species concomitant with energy conservation, as opposed to the assimilatory reduction of the same oxyanions to ammonia for biosynthetic purposes. Dissimilatory and assimilatory processes are juxtaposed in Table 1. The dissimilatory branch comprises ammonification in addition to denitrification, and both processes are initiated by respiratory nitrate reduction. Ammonification is the reduction of nitrate to ammonia that does not serve the purpose of N autotrophy. Sometimes the term is used to describe the liberation of ammonia from an organic molecule or even for the conversion of N_2 to ammonia (N_2 fixation), but those are the less preferred choices.

A bacterium is either denitrifying or ammonifying; apparently there is no option within the cell to proceed either way. The ammonifying pathway is mostly not electrogenic, detoxifies nitrite, and serves as an electron sink. Ecological, physiological, biochemical, and genetic aspects of ammonification have been covered elsewhere (153, 820).

Denitrification will be considered here as the assemblage of nitrate respiration, nitrite respiration combined with NO reduction, and N_2O respiration:

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$$

NO has recently been proven to be an intermediate (354, 940, 966). Both N_2O and NO fulfill the criteria expected from obligatory and free intermediates, i.e., kinetic competence, the possibility to feed the intermediates as precursors into the process and to detect them upon chemical or mutational blockage of the pathway, and biochemical and genetic evidence for the enzymes responsible for metabolizing these intermediates.

The physiology and regulation of NO metabolism are closely interlaced with nitrite reduction (see the section on regulation, below). Whether NO reduction is able to sustain a respiratory process in its own right, independent of nitrite reduction, requires further investigation. Even in a complete denitrifier, the three respiratory complexes maintain a certain degree of independence since they respond to combinations of different external and internal signals. The entire process is thus best described as of modular organization. When it is possible to

TABLE 2. Genes for the denitrification process and their functions

Category of affected process	Gene or locus[a]	Mol mass (kDa) of gene product[b]	Encoded gene product, function, or observation	Reference(s)
Regulation	*anr*	27.1	*P. aeruginosa* FNR-like global redox regulator for expression of denitrification genes	264, 721
	dnr, *fnrD*	24.5–26.2	*Pseudomonas* FNR-like regulators; affect the expression of *nirS* and *norCB*	22, 882
	$fixK_2$		*B. japonicum* FNR-like regulator; affects anaerobic growth on nitrate	249
	fnrP	28	*Paracoccus* FNR-like regulator; affects the expression of *narGH*	871
	narL	24.4	Nitrate-responsive transcription factor of *Pseudomonas* of a NarXL two component system	320
	nirI	73.1	A membrane protein with similarity to NosR; affects *nirS* expression	184
	nirR	25.6	*Pseudomonas* locus; affects NirS synthesis	429
	nirY (*orf286*)	32.7	LysR-like regulator	283
	nnr, *nnrR*	26	*Paracoccus* and *Rhodobacter* FNR-like regulators; affect *nirS* and *norCB* expression	833, 870
	nnrS		Activates transcription of *nirK* and *nor* genes in *R. sphaeroides*	496
	nosR	81.9	Membrane-bound regulator required for transcription of *nosZ*	171, 172, 349, 357, 974
	rpoN	54.8	Sigma factor σ^{54} affects denitrification; essential for *R. eutropha*	320, 893
Nitrate respiration	*narD*		Plasmid-borne locus for *R. eutropha* respiratory nitrate reduction	892
	narG	139	Large or α subunit of nitrate reductase; binds MGD	646
	narH	57.3	Small or β subunit of respiratory nitrate reductase; binds Fe-S clusters	70
	narI	26.1	Cytochrome *b* subunit of respiratory nitrate reductase	70
	narJ	25	Protein necessary for nitrate reductase assembly	70, 82, 227
Periplasmic nitrate reduction	*napA*	92.6–93.3	Large subunit of periplasmic nitrate reductase; binds MGD and Fe-S cluster	71, 762
	napB	17.8–18.9	Small subunit of periplasmic nitrate reductase; a diheme cytochrome *c*	71, 762
	napD	12.1	Cytoplasmic protein with presumed maturation function; homologous to *E. coli* NapD (YojF)	71, 303, 762
	napE	6.6	Putative monotopic membrane protein; no known homologs	71
Nitrite respiration	*nirB*	30.4	Cytochrome c_{552}	430
	nirC	11.9	Monoheme cytochrome *c* with putative function in NirS maturation	430, 938
	nirK, *nirU*	36.9–41	Cu-containing nitrite reductase	144, 282, 608, 826, 832, 941
	nirN, *orf507*	55.5	Affects anaerobic growth and in vivo nitrite reduction; similarity to NirS	283, 444
	nirQ	29.2	Gene product affects catalytic function of NirS and NorCB	21, 431
	nirS (*denA*)	62	Cytochrome cd_1 nitrite reductase	185, 430, 620, 672, 770
Heme D_1 biosynthesis	*nirD*	16.9	Gene product affects heme D_1 biosynthesis or processing	185, 429, 444, 634
	nirE	29.6	*S*-Adenosyl-L-methionine:uroporphyrinogen III methyltransferase	185, 283, 444
	nirF	43.1	Required for heme D_1 biosynthesis or processing; similarity to NirS	185, 443, 634
	nirG	16.6	Gene product affects heme D_1 biosynthesis or processing	444, 634
	nirH	18.8	Gene product affects heme D_1 biosynthesis or processing	444, 634
	nirJ, *orf393*	44.4	Affects heme D_1 biosynthesis or processing; similarity with PqqE, NifB and MoaA	283, 444
	nirL	19.6	Gene product affects heme D_1 biosynthesis or processing	444, 634

Continued on following page

TABLE 2—*Continued*

Category of affected process	Gene or locus[a]	Mol mass (kDa) of gene product[b]	Encoded gene product, function, or observation	Reference(s)
NO respiration	*norB*	52–53.1	Cytochrome *b* subunit of NO reductase	23, 39, 186, 968
	norC	16–17	Cytochrome *c* subunit of NO reductase	23, 39, 186, 968
	norD, *orf6*	69.7	Affects viability under denitrifying conditions	23, 39, 186
	norE, *orf2*, *orf175*	17.7–19.5	Membrane protein; homologous with COX III	21, 186, 283
	norF	8.2	Affects NO and nitrite reduction	186
	norQ	30.5	Affects NirS and NorCB function; homolog of NirQ	39, 186
	norZ	84.5	Chromosomally encoded *R. eutropha* NO reductase	163
N_2O respiration	*fhp*	44.8	*R. eutropha* flavohemoglobin affects N_2O and/or NO reduction	162
	nosA, *oprC*	74.9–79.2	Channel-forming outer membrane protein; affects Cu-processing for NosZ	508, 945
	nosD	48.2	Periplasmic protein involved in Cu insertion into NosZ	349, 357, 974, 984
	nosF	33.8	ATP/GTP-binding protein involved in Cu insertion into NosZ	357, 984
	nosL	20.4	Part of *nos* gene cluster; putative outer membrane lipoprotein	134, 224
	nosX	34.1	Affects nitrous oxide reduction in *S. meliloti*	134
	nosY	29.4	Inner membrane protein involved in Cu processing for NosZ	357, 984
	nosZ	70.8	Nitrous oxide reductase	138, 349, 357, 878, 974
Electron transfer	*azu*	16	Azurin	30, 117, 352, 928
	cycA	11.7–15.5	Cytochrome c_2 (c_{550})	96, 216, 872
	napC	27.2	Tetraheme cytochrome *c*; homologous to NirT	71, 762
	nirM (*denB*)	10.8	Cytochrome c_{551}	25, 430, 613
	nirT	22.8	Putative membrane-anchored tetraheme *c*-type cytochrome	430
	paz	15.7	Pseudoazurin	144, 928
Functionally unassigned	*orf396*	43.1	A putative 12-span membrane protein of *P. stutzeri*; homologous to NnrS	283
	nirX	32.4	A *Paracoccus* putative cytoplasmic protein; homologous to NosX	184, 674
	orf7, *orf63*	7.3	*Pseudomonas* genes immediately downstream of *dnr* and *fnrD*	22, 882
	orf247	25.8	Putative member of the short-chain alcohol dehydrogenase family	283

[a] Mnemonics for gene designations: *azu*, azurin; *anr*, arginine nitrate regulation; *cyc*, cytochrome *c*; *dnr*, dissimilatory nitrate respiration regulator; *fhp*, flavohemeprotein; *fix*, nitrogen fixation; *fnr*, fumarate and nitrate respiration; *nap*, nitrate reductase, periplasmic; *nar*, nitrate respiration; *nir*, nitrite respiration; *nnr*, nitrite and nitric oxide reductase regulator; *nor*, nitric oxide respiration; *nos*, nitrous oxide respiration; *paz*, pseudoazurin; *rpo*, RNA polymerase.

[b] Molecular masses of unprocessed gene products without cofactors.

distinguish the individual process, use of the specific over a more global term is preferred. For example, the qualifier "dissimilatory" does not distinguish between the respiratory or ammonifying modes of nitrate reduction; note also that seen as isolated processes, neither nitrate respiration nor N_2O respiration is denitrifying in the original sense of the term.

Trace gas metabolism depending on nitrate or nitrite and usually yielding N_2O without having bioenergetic significance is observed for both the ammonifying and assimilatory branches of nitrate reduction and is usually referred to as denitrification sensu lato (83, 775, 776, 947). In the ecophysiological context, the possibility of chemodenitrification, i.e., the nonenzymatic conversion of nitrate or nitrite to a gas species, has to be considered.

Organismic diversity is an important aspect of denitrification and encourages comparative and evolutionary interpretations. This requires a consolidated taxonomic basis upon which to formulate common principles. New names or modifications of familiar ones reflect the progress in clarifying the systematic relationship among the denitrifying bacteria. Relevant nomenclatorial transfers of denitrifying taxa are tabulated in the final section, and the currently valid names are used throughout this article. Invalid taxa, following convention, are placed in quotation marks.

The N oxide species $NO^{\bullet}$ and N_2O are commonly termed nitric oxide and nitrous oxide in the denitrification literature, based on the continuing practice to amend the more highly oxidized, oxygen-rich species with the suffix -ic and the more highly reduced one with the suffix -ous. International Union of Pure and Applied Chemistry nomenclature rejects this convention and recommends the terms "nitrogen monoxide" for $NO^{\bullet}$ and "dinitrogen monoxide" for N_2O; these terms, although more accurate than the trivial names, have not yet found acceptance. $NO^{\bullet}$ is a radical, but for simplicity will be written below without the radical designation.

Nitrification is a property of both chemolithotrophic and heterotrophic bacteria. The expression "heterotrophic nitrification" is used here to describe the oxidation of ammonia associated with organotrophic metabolism to contrast it with the better-known variant of autotrophic nitrification. Note,

however, that the same term has been used to describe the conversion of reduced nitrogen in amines, oximes, hydroxamates, and other N compounds to a higher oxidation state of nitrogen or the liberation of nitrate and nitrite.

GENETIC BASIS OF DENITRIFICATION

Access to denitrification genes was first sought by conjugational and transductional mapping in *Pseudomonas aeruginosa*. An important outcome of the early genetic analysis was the finding that *P. aeruginosa* encodes the respiratory (*nar*) and the assimilatory (*nas*) nitrate reductase systems from distinct gene sets (761). This was also shown for *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) (892) and the nitrate respirer *Klebsiella pneumoniae* (515, 901) and is assumed to be the rule for nitrate-assimilating denitrifiers. The distinct genetic basis for the respiratory and assimilatory process manifests itself in regulatory responses. Genes for nitrate assimilation are repressed by ammonia and do not respond to oxygen, while the expression of *nar* genes occurs at low oxygen concentrations and does not respond to ammonia. Oxygen inhibits nitrate uptake for nitrate respiration of denitrifiers or nitrate respirers (335) but has no effect on nitrate assimilation.

Genes encoding the five N oxide reductases of denitrification have been identified from random transposon Tn*5* mutagenesis, complementation analysis, and screening of cosmid and expression libraries. The genes currently identified as associated with the process are listed in Table 2. Once the analysis of the nitrate-reducing system of denitrifiers has advanced to the level achieved with enterobacteria, the total number of genes necessary for denitrification might well increase to about 50 for a single organism.

Gene Clusters and Nature of Denitrification Genes

The genes for denitrification encoding functions for nitrate respiration (*nar*), nitrite respiration (*nir*), NO respiration (*nor*), and N_2O respiration (*nos*) are assembled in clusters in *Pseudomonas stutzeri* (100, 429), *P. aeruginosa* (22), *Paracoccus denitrificans* (70, 185, 186), *Sinorhizobium* (formerly *Rhizobium*) *meliloti* (357), and "*Achromobacter cycloclastes*" (556). The *nir* and *nor* genes are closely linked in the pseudomonads and in *Paracoccus denitrificans*. Figure 2 shows the comparative gene organization of the three denitrifiers for which these clusters have been analyzed to a significant extent. The *nir-nor* gene clusters harbor the structural information for both reductases and the functions for metal processing, cofactor synthesis, electron donation, protein maturation, assembly processes, and regulation. So far only in *P. stutzeri* are the *nos* genes linked with the *nir* and *nor* genes, forming a supercluster of about 30 kb comprising 33 genes. Clustering of *nir* and *nor* functions may be common among denitrifiers, at least those depending on cytochrome cd_1 nitrite reductase, and may facilitate genetic analysis.

In the following, a brief characterization of genes in categories of the function of the gene products is given (Table 2). The isolation of genes for N_2O utilization marked the beginning of the current stage of genetic analysis. The *nos* gene cluster of about 8 kb was found by mapping Tn*5* mutations in mutants defective in N_2O reductase expression or processing. *nosZ*, the structural gene for N_2O reductase, was identified by heterologous expression in *Escherichia coli* and was the first denitrification gene of known structure (878). The *nosZ* probe derived from *P. stutzeri* hybridized with homologous genes of *P. aeruginosa* (974), *R. eutropha* (974), *Paracoccus denitrificans* (349), and *S. meliloti* (357), indicating a high degree of conservation among N_2O reductases, which was confirmed by sequencing these genes.

The *nirS* gene for cytochrome cd_1 nitrite reductase was first isolated from *P. aeruginosa* with oligonucleotide probes designed from the amino acid sequence of the purified enzyme (770). It was found independently by targeting *nirM*, the gene for cytochrome c_{551}, which is located in *P. aeruginosa* just downstream of *nirS* (25, 613). The cytochrome cd_1 genes from *P. stutzeri* ZoBell and JM300 were isolated with a phage expression library and protein-deduced oligonucleotide probes, respectively (430, 774).

The structural gene, *nirK*, for the Cu-containing nitrite reductase (originally also termed *nirU* or *nir*) was identified in *Pseudomonas* sp. strain G-179 by targeting the wild-type gene with Tn*5* (941), in *Alcaligenes faecalis* S-6 by using protein-derived oligonucleotides as screening probes (608), and in *Pseudomonas aureofaciens* by screening an expression library with an anti-NirK antiserum (282).

The *norCB* genes encoding the NO reductase complex were found by screening a cosmid bank of *P. stutzeri* with an oligonucleotide probe derived from the N terminus of the purified NorC subunit (99). The *nor* genes were shown by cosmid mapping to be closely linked to *nirS* (429). Anticipating a similar clustering of *nir* and *nor* in *P. aeruginosa* (23) and *Paracoccus denitrificans* (186), homologous *norCB* genes were identified by sequencing *nirS*-carrying DNA fragments.

By now, a small pool of primary structures of the reductases from various sources is known. The sequence relationship among the denitrification enzymes is depicted in phylogenetic unrooted trees in Fig. 3. NirS and NosZ sequences form relatively tight clusters, whereas the plasmid-encoded NorB sequence of *R. eutropha* is somewhat removed from its homologs. While the NorB proteins and NosZ proteins of the pseudomonads are found at neighboring branches of their respective trees, the pseudomonadal NirS proteins are separated. This suggests an evolution of NirS independent from the other reductases. Also on the basis of other arguments (see the section on N_2O respiration, below), the utilization of N_2O is conceived as a separate respiratory system. The NirF and NirN proteins, which are presumed to be paralogous with NirS, are found on separate lineages on the NirS tree and are only distantly related to NirS. The NirK proteins show no well-defined clustering. The NirK proteins of *Rhodobacter sphaeroides* and *P. aureofaciens* evolved along a separate lineage and are distant both from the rest of the NirK proteins and from each other.

The *nar* genes for respiratory nitrate reduction are not linked to the denitrification genes proper in *P. aeruginosa* and *P. stutzeri*. The structural genes for the nitrate reductase complex, *narGHJI*, have been sequenced from the nitrate respirers *E. coli* (79) and *Bacillus subtilis* (168, 350) but remain to be completed for the first example of a denitrifier (70, 646).

The structural genes encoding the two subunits NapA and NapB of the periplasmic nitrate reductase were first identified by screening a cosmid library of the pHG1 megaplasmid of *R. eutropha* with oligonucleotides derived from the N termini of the purified subunits (762). The two structural genes were sequenced, and the basic properties of this enzyme derived. A more extended *nap* locus, comprising *napEDABC*, was cloned and sequenced from a genomic cosmid library of *Paracoccus denitrificans* GB17 (71). The probe for screening was based on gene amplification from internal peptide sequences of the purified NapA subunit. Homologs of *napA* have been detected by hybridization in *P. aeruginosa* and *P. stutzeri*; in both cases, these genes are not linked to the *nar* genes (883).

Different types of regulators are encoded within the *nir-nor*

FIG. 2. Comparative organization of denitrification genes among cytochrome cd_1-synthesizing denitrifiers. The approximate size and transcriptional direction of genes are given by the arrow boxes. Homologous genes within the *nir* and *nor* regions are shown by the same graphic patterns; open boxes have no homologs. Solid boxes represent the structural genes for N_2O reductase (*nosZ*), nitrite reductase (*nirS*), and NO reductase (*norCB*). Boxes with broken outlines denote genes that are only partially sequenced. The gene clusters are aligned with respect to the position and transcriptional orientation of *nirS*. For sequence sources, see Table 2.

clusters by the genes *nnr*, *dnr*, *fnrD*, *nosR*, *nirI*, and *nirY*. The genes *nosR* and *nirI* encode putative regulators for *nosZ* and *nirS* expression, respectively, and are predicted to possess iron-sulfur centers (see the section on regulation, below). *nnr*, *dnr*, and *fnrD* are each found downstream of and in the opposite direction to the *nor* operons (Fig. 2). The encoded proteins belong to the FNR family of transcription factors (22, 870, 882). Regulatory genes for denitrification belonging to the FNR family also reside outside the known denitrification loci. *anr* of *P. aeruginosa* encodes a global transcriptional activator for anaerobic metabolism including denitrification (264). The structurally although not functionally homologous gene *fnrP* of *Paracoccus denitrificans* maps adjacent to *cco*, which encodes a cytochrome cbb_3-type oxidase (187, 871). This is also the case for the *anr* homolog *fnrA* of *P. stutzeri* (882); however, this gene does not affect denitrification directly.

In *Rhodobacter sphaeroides*, the *nir* and *nor* regions are not closely linked on the chromosome. Instead, they form a regulon under the common control of NnrR, another member of the FNR family (497). The *nnrR* gene is located there immediately upstream of *norCB* and is transcribed in the same direction. The *narXL* genes, which encode a two-component system and mediate the nitrate response, are part of the *narG* locus in *P. stutzeri* (320).

The intergenic region of the *nos* and *nir* operons of *P. stutzeri* harbors *nirY* (= *orf286*), whose deduced product is similar to LysR-type regulators (283). The LysR family is distinguished by a consensus DNA-binding motif in the N-terminal domain and comprises factors that control a broad variety of processes including the oxygen stress regulon (725). *nirY* is part of a gene region encoding components of heme D_1 synthesis and processing of cytochrome cd_1, but the target of its product is unknown.

To assign an electron donor to its cognate reductase will require genetic evidence. At present, only a small number of structural genes for electron carriers have been identified. *nirM*, immediately downstream of *nirS* of *P. aeruginosa*, encodes cytochrome c_{551}, an electron donor for NirS (613). *P. stutzeri* harbors the gene sequence *nirSTBM*, of which *nirM* is homologous to the *P. aeruginosa* gene and *nirT* encodes a tetraheme cytochrome with a putative electron donor function (430). The relationship between the *nirT* and *nirM* products with respect to electron donation to NirS is unknown. *nirT* is cotranscribed with *nirS* in *P. stutzeri*, which indirectly supports the view of a function in nitrite reduction (319). *nirT* and *nirB* have yet to be identified in other denitrifiers; neither gene is part of the currently analyzed gene clusters of *P. aeruginosa* and *Paracoccus denitrificans* (Fig. 2). However, *nirT* is similar to *napC*, encoding the putative electron-transferring cytochrome *c* for the periplasmic nitrate reductase (see the following section). Several homologs of *nirT* are present in nondenitrifiers, thus ascribing a broader importance to this gene (66, 116, 213, 560).

Genes encoding electron donors are not necessarily part of the denitrification gene clusters. The *cycA* gene encoding cytochrome c_{550} of *Paracoccus denitrificans* lies adjacent to the isogene for the subunit I of COX (872). For the genes encoding the electron carriers for nitrite reductase, azurin (*azu*) and pseudoazurin (*paz*), no link to a denitrification gene has yet been recognized.

Genes for ancillary functions in denitrification were found on sequencing the vicinities of the structural genes for the

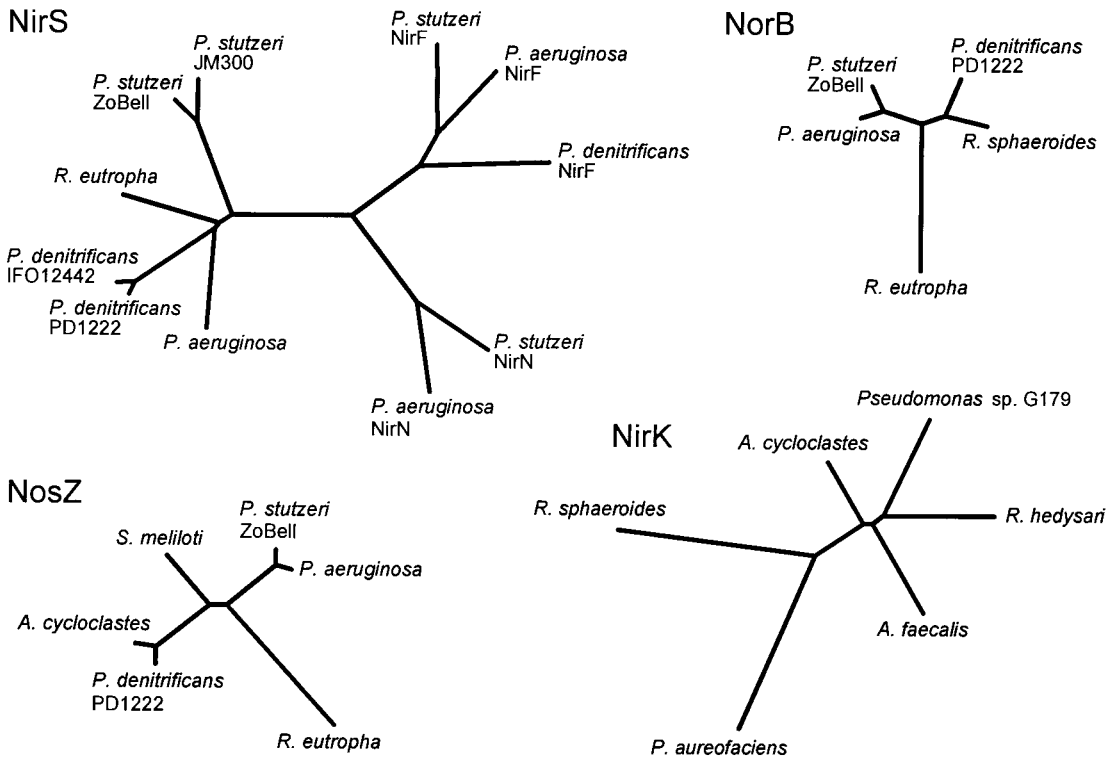

FIG. 3. Sequence relatedness among the terminal oxidoreductases of denitrification. Unrooted phylogenetic trees are as follows: NirS, cytochromes cd_1 and related NirF and NirN proteins; NorB, cytochrome *b* subunits of NO reductases; NosZ, N_2O reductases; NirK, CuNIR species. The trees were constructed with CLUSTAL W software (814) and PHYLIP software, release 3.5 (243). The sequence sources are listed in Table 2; the plasmid-encoded NorB sequence of *R. eutropha* was used without the N-terminal extension (163).

reductases. Heme D_1 is the cofactor of denitrifiers that depend on the cytochrome cd_1 nitrite reductase. Genes for heme D_1 biosynthesis are part of the denitrification gene clusters in the three organisms studied. The first gene with a likely function in heme D_1 biosynthesis, *nirE*, was found immediately downstream of *nirS* in *Paracoccus denitrificans* (Fig. 2). The derived protein shows high similarity to methyltransferases acting on uroporphyrinogen III (185). The genes for heme D_1 biosynthesis are distributed in *P. stutzeri* over two loci, *nirJEN* and *nirCFDLGH*, which lie upstream and downstream of *nirS*, respectively (283, 634). The same genes of *P. aeruginosa*, *nirCFDLGHJEN*, are clustered in one locus downstream of *nirS* and may encode the entire set of proteins required for the reaction steps leading to heme D_1 (444).

Genes encoding functions for metal processing, protein assembly, or maturation represent a further category of recognized denitrification genes (Fig. 2). Of Tn*5*-derived mutants defective in N_2O respiration, a distinct group synthesized an apo-N_2O reductase only. The affected genes of these mutants, *nosDFY*, encode a metal insertion apparatus for the reductase (984). Homologous genes have been found in other denitrifiers. In *P. aeruginosa* (974) and *Paracoccus denitrificans* (349), *nosD* genes also exist downstream of *nosZ*. In more extended analyses, *nosRZDFY* sequences have been identified in "*A. cycloclastes*" (556) and *S. meliloti* (357), suggesting a high degree of organizational conservation. In *R. eutropha*, these ancillary genes have not yet been identified since they are not located in the immediate vicinity of *nosZ* (974).

The functions of the genes *nosL* and *nosX* (homologous to *nirX*) are still insufficiently understood. The *nosL* product is tentatively considered an outer membrane protein featuring the signal peptide of a lipoprotein (224). The signature of a disulfide isomerase deduced from the *P. stutzeri* protein is not present in NosL of "*A. cycloclastes*" (556) and *S. meliloti* (134), which invalidates the original proposal and requires a new functional assignment. Unfortunately, a *nosL* mutation in *P. stutzeri* is phenotypically silent.

nosX is required for N_2O utilization by *S. meliloti* in an as yet unspecified function (134). The gene is also part of the *nos* region of "*A. cycloclastes*" (556). The homolog *nirX*, with ≈36% sequence identity, has been found as part of the *nir* gene region of *Paracoccus denitrificans* (Fig. 2) (674). The gene is absent from the *nos* and *nir* gene clusters of *P. stutzeri* and was also not picked up by random mutagenesis (877, 972). It is very likely that there will be denitrification genes specific for a distinct species or for groups of denitrifiers; yet it would be premature to pinpoint those candidates from the limited genetic information available.

The *orf2* product of *P. aeruginosa* and its homologs (the *orf175* product and NorE of *P. stutzeri* and *Paracoccus denitrificans*, respectively) are structurally related to subunit III of COX, and an analogous role is feasible (21). Given the similarity of the cytochrome *b* subunit of NO reductase to subunit I of COX, a function for the *orf2* product in NO reduction does not seem unlikely (186, 978).

The *nirQ* gene, located upstream of *nirS* in the pseudomonads (and its homolog *norQ*, located downstream of *norB* in Paracoccus denitrificans) may code for a maturation or assembly function for a denitrification enzyme. Mutations of *nirQ* or *norQ* affect both NO reduction and nitrite reduction (186, 431). The immediate target gene(s) or reaction partners of the *nirQ* product are not known. Because of the sequence similar-

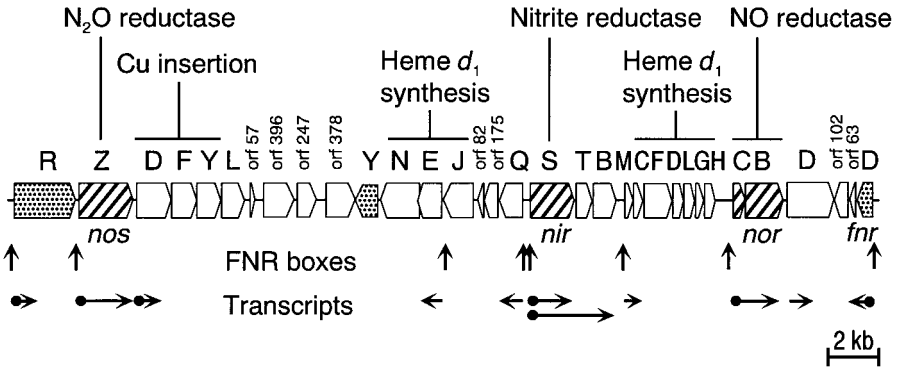

FIG. 4. Denitrification gene cluster of *P. stutzeri* and its transcriptional organization. The direction of transcription is shown by arrow boxes. Representative coding functions for groups of genes are indicated; for others, see Table 2. Unassigned ORFs are given by the number of amino acids of the derived gene products. Striped boxes denote the structural genes for the indicated N oxide reductases; dotted boxes represent genes with a regulatory function. Vertical arrows show the locations of putative recognition motifs for an FNR-like transcription factor. Horizontal arrowheads mark transcript initiation sites; mapped promoters are indicated by a dot. Extended arrows represent the experimentally identified transcripts of *nosZ*, *nirSTB*, and *norCB*.

ity of NirQ to regulators and because of a putative nucleotide-binding region and the pleiotropic nature of a *nirQ* mutation, a regulatory role has also been considered. Further *nirQ* homologs are *cbbQ* and *gvpN*, which are part of the genes for C autotrophy of *Pseudomonas hydrogenothermophila* (944) and gas vesicle formation of *Anabaena flos-aquae* (454), respectively; partial sequence information indicates the presence of a *nirQ* homolog in *Chromatium vinosum* also (876). Thus, members of the NirQ protein family certainly must have varied functions affecting other processes besides denitrification.

The presence of several more genes is indicated from uncharacterized proteins whose expression is denitrification dependent. Periplasmic (35-kDa) and cytoplasmic (32-kDa) proteins are induced by nitrous oxide (or nitrate) in *Rhodobacter sphaeroides* IL106. Neither protein reveals similarity to known denitrification components based on partial amino acid sequences (707). Under denitrifying conditions, certain *nosA* mutants of *P. stutzeri* JM300 express a soluble protein (NosE $\approx$70 kDa) of unknown function (588). The *nosA* gene encodes an outer membrane protein that was recognized from its property as a phage receptor (150). *nirR* of *P. stutzeri* affects nitrite reduction (429). The gene is located outside the denitrification gene cluster and encodes a protein of 25.6 kDa that has no noteworthy similarity to known proteins in data banks (unpublished data).

Genetic Organization and Gene Expression

Of the 33 genes in the denitrification cluster of *P. stutzeri*, 23 are transcribed in the same direction; only two groups, comprising 3 and 7 genes, are transcribed oppositely (Fig. 4). Ten transcriptional units can currently be defined with confidence, but since not all promoters have been mapped, the definitive number will be greater. The *nir-nor* gene clusters of *Paracoccus denitrificans* (186) and *P. aeruginosa* (24) each comprise currently about four or five recognizable transcriptional units. The 11 genes *nirS* through *nirN* of *P. aeruginosa* (Fig. 2) are claimed to be transcribed as a single operon (444). Other presumed operons are the gene sequences *nirQ-orf2-orf3* from *P. aeruginosa* and *norCBQDEF* of *Paracoccus denitrificans*. Polycistronic transcripts have been identified experimentally so far only for *norCB* and *nirSTB* of *P. stutzeri* (319, 968).

Several genes of the *nir* region in the pseudomonads appear to be duplicated. The products of *nirF* and *nirN* both have sequence similarity to NirS, including a heme-binding motif in NirN (283, 444). They may be members of a paralogous gene family. Mutational evidence shows that NirF and NirN do not function as nitrite reductases. The *nirDLGH* products show pairwise similarity indicative of two putative gene duplication events (444, 634).

Identification of promoters and the transcriptional organization within the denitrification gene cluster allow first generalizations. Transcript initiation sites have been mapped for *azu* (30); *nosR*, *nosZ*, and *nosD* (171); *norCB* (968); *nirS* (319); and *cycA* (792). Even among this very small number, it was found that certain genes are transcribed from more than one promoter. Denitrification is an environmentally regulated process, and the use of multiple promoters may be a means of adjusting gene expression differentially with respect to the oxygen supply, the presence and nature of an N oxide, and perhaps further external factors such as metal ions. It is anticipated that the promoters of principal denitrification genes are organized specifically for the integration of multiple environmental signals.

nosZ is expressed from six promoters; anaerobic expression occurs preferentially from promoter P3, whereas the weak constitutive expression observed in aerobic cells is likely to depend on promoter P2 (171). Cytochrome c_{551} (the *nirM* product) and cytochrome c_{550} (the *cycA* product) are expressed both under O_2-respiring and denitrifying growth conditions (95, 96, 518), a puzzling fact in light of their electron donor role in denitrification. Rather than resorting to two sets of genes, the existence of two promoters, as shown for *cycA* (792), provides a satisfactory explanation for the dual expression of these cytochromes. Expression of the gene for cytochrome c_2 (the *cycA* product) from *Rhodobacter sphaeroides* 2.4.1 is regulated in response to anaerobic photosynthetic and aerobic chemoheterotrophic conditions from two promoters; it is negatively affected by heme or an intermediate of tetrapyrrole synthesis (727). Strain 2.4.1, although nondenitrifying, has a nitrate reductase and an N_2O reductase (576), of which the latter depends on cytochrome c_2. It is not known whether *cycA* expression responds to anaerobic nitrate- or N_2O-respiring conditions.

Anaerobic promoter activity in *P. aeruginosa* has been detected for *nirS* by a *xylE* gene fusion (20) and for *nirS*, *nirQ*, and *norCB* by *lacZ* fusions (22). All upstream regions of the sequenced *nirS* and *norC* genes exhibit binding motifs for an FNR-like factor, which suggests the presence of anaerobically controlled promoter structures. The FNR recognition sites in the promoters of denitrification genes are probably recognized

by the members of the FNR family mentioned above (see also the section on regulation, below).

Heterologous expression of genes for site-directed mutagenesis or biotechnological applications will have to reckon with diverse regulatory and maturation requirements of denitrification components. For instance, a lack of ancillary genes results in nonfunctional cytochrome cd_1 and N_2O reductase on heterologous expression of *nirS* and *nosZ* in *Pseudomonas putida* and *E. coli*, respectively (282, 767, 878). Genes for the blue copper proteins nitrite reductase (282, 608), azurin (137, 438, 860), and pseudoazurin (144, 928) are expressed as metal-containing holoproteins in *E. coli*. Either ancillary functions for metalloprotein biogenesis are provided by the host or Cu is incorporated spontaneously. Azurin can be expressed from pUC vectors to comprise ≈27% of the total protein in the periplasmic space. The material contains an inactive, probably chemically modified variant of azurin lacking Cu-binding properties (860). Only recently was *E. coli* shown to have a periplasmic, Cu-containing SOD (41, 64). This opens the possibility that the biosynthesis of a foreign periplasmic Cu protein in *E. coli* takes advantage of the innate Cu-processing capabilities of the host cell.

Expression of *nirK* in *E. coli* at 37°C yielded only insoluble material within inclusion bodies. At 27°C, some soluble material was produced but still the enzyme was not processed correctly and exported, since several immunoreactive peptides showed up on SDS-electrophoresis of the cytoplasmic fraction (488). A construct from which a high-activity enzyme was expressed and exported into the periplasm required truncation of the native export signal by seven amino acids, an increase of the N-terminal charge by 1, and an increase of the overall hydrophobicity of the signal peptide. With this construct the successful expression of native and recombinant nitrite reductase was possible (488, 490). Recombinant NosZ proteins have been expressed in a *nosZ* deletion mutant from a pSUP vector (973).

In contrast to the by now sizable number of products of known denitrification genes, few gene products have been isolated. In addition to the reductases, only cytochrome c_{551}, cytochrome c_{550}, cytochrome c_2, cytochrome c_{552} (a diheme protein), azurin, and pseudoazurin have been purified. Gene products involved in a regulatory function, assembly, or biosynthetic reactions have yet to be isolated and characterized biochemically.

Genome Size and Gene Maps of Denitrifiers

The advances in generating and handling macrorestriction fragments have led to chromosomal maps for several denitrifying bacteria and to accurate assessments of their genome size. Genomes among denitrifying genera vary within a wide range. The highest and lowest extremes are represented by *Bradyrhizobium japonicum* (8.7 Mb) (493) and *Neisseria gonorrhoeae* (2.2 Mb) (195), respectively. Comparative data are available for pseudomonads to show that genome size varies considerably within this genus. The range extends from 2.6 to 6.8 Mb for *Pseudomonas lemoignei* and *Pseudomonas glathei*, respectively (neither species denitrifies). The genomes of *P. aeruginosa* PAO (5.9 Mb) (356), *P. aeruginosa* C (6.5 Mb) (731), and *P. fluorescens* SBW25 (6.63 Mb) (664) are at the upper end of this range. A determination of the genome size of 20 strains of *P. stutzeri* with the rare-cutting restriction enzyme *Ceu*I, which results in only four DNA fragments, gives a range of 3.7 to 4.6 Mb (278). A value of 4.3 Mb was extrapolated for the ZoBell strain.

The most detailed chromosomal map of a denitrifying bacterium is that of *P. aeruginosa* PAO, which comprises close to 200 identified markers; among them are about a dozen related to anaerobic nitrate respiration and denitrification (356). Genes for denitrification have been recently mapped to find whether they are organized as an "anaerobic respiration island" that could propagate this facultative trait by lateral gene transfer among the prokaryotes (883). Although the genes are concentrated in the 20- to 36-min segment of the chromosome, they are organized as individual clusters harboring genes for nitrite and NO reduction (19.9 to 20.5 min), separate from those for nitrate reduction (27.2 to 28.4 min) and N_2O reduction (33.7 to 35.8 min) (Fig. 5). Only the *nir* and *nor* genes are commingled to come close to an anaerobic respiration island. However, it should also be noted that this appears to be limited to denitrifiers that depend on cytochrome cd_1, whereas in the copper nitrite reductase-containing denitrifiers the *nir* and *nor* genes are separated (39, 832). The *anr* gene for the anaerobic global regulator maps independently from denitrification genes at 60 min (264, 883). Further separate loci comprise the genes for the outer membrane protein NosA, the periplasmic nitrate reductase (*nap*), and azurin (*azu*). The *hemL* (372) and *hemA* (373) genes, necessary for anaerobic heme biosynthesis, map at 0.3 to 0.9 and 24.1 to 26.8 min, respectively. Genes involved in heme D_1 biosynthesis, with *nirE* as the marker gene, are part of the *nir* locus (Fig. 5).

Several more loci dispersed over the chromosome and associated with nitrate utilization have been mapped by classical techniques. These loci require further analysis to reveal their encoded functions (Fig. 5). Only the loci *nir-9006* (21 min) and

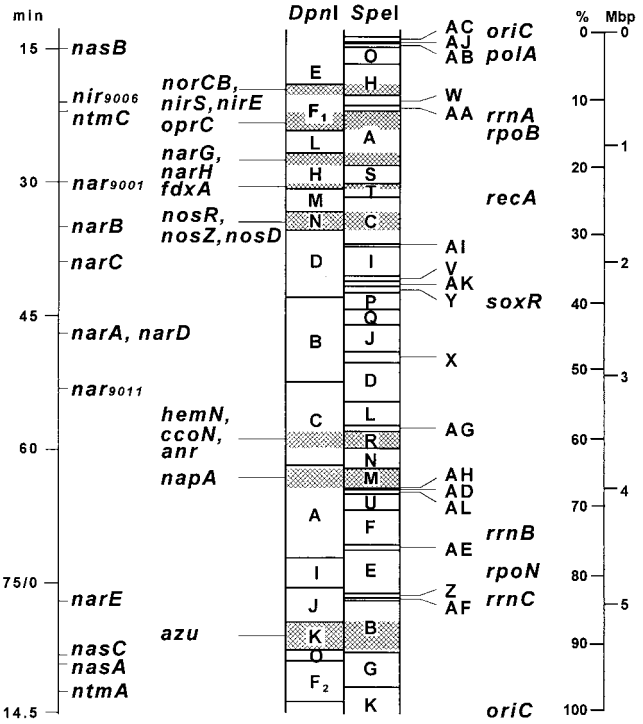

FIG. 5. Physical map of the *P. aeruginosa* PAO linearized chromosome with gene locations for inorganic nitrogen metabolism. Shaded segments indicate the locations of denitrification genes deduced from *Spe*I and *Dpn*I macrorestriction fragment analysis (883). For discussion of the denitrification loci, see the text; *fdxA* is the locus for an anaerobically expressed ferredoxin (unpublished data). The loci for nitrate metabolism mapped by classical genetic techniques are given with their approximate position on the time scale (minutes). A few markers are shown on the right-hand side for orientation (356). The map coordinates are from references 698 and 699.

nar-9001 (30 min) are close enough to the *nirS* and *narGH* loci to make identity feasible. The *nar-9011* locus affects the reduction of nitrate to nitrite (355); *narA*, *narB*, *narC*, *narD*, and *narE* affect anaerobic growth on nitrate (405). For *narD*, a role in molybdenum transport or processing for nitrate reductase is suggested, since the respective gene defect is suppressed by a high dose of molybdate (867). *ntmA* and *ntmB* are loci that affect growth on distinct sources of nitrogen. Functions required for assimilatory nitrate reduction, *nasABC*, have been mapped at three loci: *nasC* was suggested to harbor the structural gene for the assimilatory reductase, and *nasAB* was related to molybdenum cofactor biosynthesis. Genes for the assimilatory nitrate reductase have not yet been isolated from any denitrifier.

Chromosome maps with a limited number of markers have been generated for *Bacillus cereus* (122), *Bradyrhizobium japonicum* (493), *S. meliloti* (360), *N. gonorrhoeae* (195), *Neisseria meningitidis* (263), *P. fluorescens* (664), *Rhodobacter sphaeroides* (800), and *Rhodobacter capsulatus* (251) and for the archaeon *Haloferax mediterranei* (genome size, 2.9 Mb) (17). They provide a basis for locating denitrification genes on these maps. Genome sequencing projects are under way for *N. gonorrhoeae*, *N. meningitidis*, *P. aeruginosa*, and the archeon *Pyrobaculum aerophilum*. Eventually, this will allow us to test hypotheses about horizontal gene transfer and the evolutionary relationship among the denitrifiers.

A remarkable case is represented by *Rhodobacter sphaeroides* 2.4.1 in its possession of two circular chromosomes (3 and 0.9 Mb) in a 1:1 stoichiometry in addition to several plasmids (801). The distribution of denitrification genes with respect to both chromosomes represents an interesting problem. Strain 2.4.1, although nondenitrifying, has a nitrate reductase and an N_2O reductase that cross-react with antibodies raised against the *Rhodobacter sphaeroides* IL106 proteins (576). Its nondenitrifying property is apparently due to an inability to reduce nitrite because the structural gene for nitrite reductase, *nirK*, is missing (496, 832). The chromosome structure of strain IL106 is unknown. A 108-kb plasmid found in this strain is not thought to be of importance for denitrification (576).

Denitrification has a sporadic but recurrent record of being an unstable trait (404, 965), yet a frequent plasmid location of denitrification genes, which could explain this instability, is not the case. The well-studied sources *P. aeruginosa*, *P. stutzeri*, and *Paracoccus denitrificans* have their denitrification genes located chromosomally. The 0.45-Mb plasmid pHG1 for chemolithotrophic growth of *R. eutropha* H16 on hydrogen has for a long time been the paradigm for plasmid-encoded denitrification (696, 732). However, even there the principal denitrification genes *nar*, *nirS*, and *norZ* are located on the chromosome (163, 672), whereas *nosZ*, *norB*, the *nap* genes, and certain supporting functions for anaerobic growth are found on the megaplasmid (762, 763, 974). On transfer of pHG1 to other hosts, it is maintained stably only in another strain of *R. eutropha* (732). A second case of plasmid-borne denitrification genes was found with *S. meliloti* (357), where the 1.4-Mb pNOD plasmid for nitrogen fixation and symbiosis carries a *nosRZDFY* cluster.

RESPIRATION OF NITRATE

The complete denitrification process leading to N_2 formation starts with the nitrate-reducing system. Knowledge about this system in denitrifiers is required to reveal the regulatory factors directed at nitrate reduction per se and those interlacing this reaction with denitrification in the strict sense. Several factors of a regulatory network combining the initiator reaction with the main process are just being recognized, and a full experimental penetration of this network is anticipated to be a long-term task.

Nitrate respiration has long been studied in *E. coli*. Given the prevalence of nitrate-respiring organisms sensu stricto and the array of available genetic tools for *E. coli*, it is not surprising that most of our knowledge about nitrate reductase has come and will continue to come from this nitrate-respiring but nondenitrifying enterobacterium. *E. coli* ammonifies nitrite with enzymes encoded by the *nir* and *nrf* systems but has no coding capability for the assimilatory nitrite reductase, a siroheme protein like the *nirBD*-derived dissimilatory nitrite reductase. The properties of the respiratory nitrate reductase of *E. coli* have been reviewed against a biochemical and genetic background (88, 273, 381). Respiratory and assimilatory nitrate reductases both have been amply covered in the context of catalysis by oxo molybdoenzymes (338). Also, the nature and function of molybdenum and iron-sulfur centers in electron transfer by the respiratory and periplasmic nitrate reductases, together with the pertinent biochemistry, have been discussed in detail, often in the context of sequence-derived predictions and hypotheses (69). As this status has not changed significantly since then, the reader is referred to these comprehensive articles.

When a denitrifying bacterium is able to assimilate nitrate, the reaction may proceed simultaneously with nitrate respiration (855). Since the first reaction step in assimilatory and respiratory nitrate utilization is identical, the question arises whether just one enzyme would catalyze both reactions. This is not the case. As discussed in the preceding section, different genes, which are distributed over several loci on the chromosomal map of *P. aeruginosa*, exist for the two processes (Fig. 5). Nevertheless, in spite of the distinct genetic basis for respiratory and assimilatory nitrate reduction, the respiratory reductase was found to serve a quasi-assimilatory function under anaerobic conditions and to provide nitrite in *nas* mutants as the substrate for nitrogen assimilation (760). This function ceases under aerobic conditions. Gene sharing between the assimilatory and the respiratory pathway may involve only a few functions, possibly related to molybdenum cofactor synthesis or to nitrate transport (287).

Since Pichinoty's pioneering contributions on bacterial nitrate metabolism, the generally held view is that of a membrane-bound respiratory reductase and of a soluble, pyridine nucleotide-dependent variant confined to the assimilatory branch of nitrate reduction. This generalization has to be modified and broadened to accommodate a new type of dissimilatory nitrate reductase. The finding by Satoh of a novel nitrate reductase activity located in the periplasm of *R. sphaeroides* IL106 and associated with a *c*-type cytochrome (715, 719) was initially rationalized in terms of peculiarities of phototrophic metabolism (554) but is now seen as a new more general aspect of dissimilatory nitrate metabolism not restricted to phototrophs. Studies from several laboratories have provided evidence for a broad distribution of this third type of nitrate reductase encoded by the *nap* genes.

Properties of Dissimilatory Nitrate Reductases

Many bacteria have more than one of the three types of nitrate reductases which comprise the soluble assimilatory-type nitrate reductase and two dissimilatory reductases, subdivided into the respiratory and the periplasmic nitrate reductases. Evidence for the coexistence of all three nitrate reductases has been provided for *R. eutropha* and *Paracoccus denitrificans* by mutational studies, gene sequencing, and, in part, isolation of these enzymes (740, 892).

TABLE 3. Properties of dissimilatory nitrate reductases (EC 1.7.99.4)

Property	Value for:					
	Pseudomonas aeruginosa	*Pseudomonas stutzeri*	"*Pseudomonas denitrificans*"	*Bacillus halodenitrificans*	*Paracoccus denitrificans*	*Paracoccus denitrificans*[a]
Mol mass (kDa)[b]	176 (260)	140 (132)	220 (230)		160	110
Subunit mol mass (kDa)	118, 64	112, 60 (45)	136, 55, 19	145, 58, 23	127, 61, 21	93, 16
Fe/M_r		13/172	13/220	5–9/226	5–7/160	
Mo/M_r		0.5–0.8/172	0.74/220	0.4–1.1/226	0.3/160	
S^{2-}/M_r		9–12/172	12/220		4–10/160	
Prosthetic groups[c]	Moco, [4Fe-4S]	MGD, FeS	Moco, FeS, heme B	Moco, 2[4Fe-4S], heme B	Moco, FeS, heme B	MGD, [4Fe-4S], heme C
Absorbance maxima						
Protein as isolated	280, 315, 415	280, 290sh[d], 410	410	411, 520	280, 400sh	
Reduced form	415	420sh	435, 535, 558	424.6, 527, 557	420, 525, 557	
EPR parameters (*g* values) of Mo(V)	1.987, 1979, 1.963	1.986, 1.978, 1.965				1.999, 1990, 1.981
K_m for NO_3^- (mM)	0.3	3.8	0.7	2.7	0.3	
Sp act[e]	2.8	27	36.4	20–35	50	50–100
pH optimum	6–7	7.2	8.0	8–8.2	7.5	
Electron donors						
In vitro	BV	MV, BV	MV, BV	Menadiol, MV	Duroquinol, MV	MV
Physiological		Cytochrome *b*	Cytochrome *b*	Cytochrome b_{557}		Cytochrome *c*
Reference(s)	119, 285	84, 255	386	449	164, 252	61, 71, 72

[a] Periplasmic nitrate reductase encoded by *napAB*; no EC number.
[b] By gel filtration or nondenaturing gel electrophoresis; values in parentheses are for secondary species present in the preparations.
[c] The molybdenum cofactor is denoted Moco when its exact nature has not been identified.
[d] sh, shoulder.
[e] Micromoles of NO_3^- per minute per milligram.

Purification and biochemical studies of membrane-bound respiratory nitrate reductase from denitrifiers have been directed at various sources, for which representative data are shown in Table 3. In addition, nitrate reductases at various stages of characterization have been isolated from the denitrifiers *Halomonas halodenitrificans*, *Thiobacillus denitrificans*, *Rhodobacter sphaeroides* IL106, *Bacillus licheniformis*, and *Bacillus stearothermophilus* (348). Some of these nitrate reductases are susceptible to proteolytic modification during isolation (84, 386), similar to the *E. coli* enzyme (191).

Respiratory nitrate reductases are complexes of two or three subunits depending on the method of isolation. The enzyme is anchored to the cytoplasmic membrane by the NarI or γ subunit and has to be solubilized by heat or detergent. The respiratory reductase is encoded by the *narGHJI* operon. *narG* encodes the large or α subunit of 112 to 145 kDa. This subunit carries molybdenum in the form of the MGD cofactor (255, 256). In the enzyme as isolated, molybdenum is detectable as a Mo(V) species via EPR by a series of resonances around $g \approx 1.9$ (Fig. 6; Table 3). The molybdenum cofactor is the active site of the reductase (338).

Nitrate reductases solubilized by heat show the featureless spectrum of an Fe-S protein with a small peak or a shoulder around 400 to 415 nm, which disappears on reduction (Fig. 6). The *narH* gene of *E. coli* encodes the small or β subunit (58 kDa) which binds three [4Fe-4S] clusters and one [3Fe-4S] cluster (79, 308). These clusters have markedly different redox potentials. Clusters 1 and 2 have potentials of +80 and +60 mV, respectively, and clusters 3 and 4 have redox potentials of −200 and −400 mV, respectively. Cluster 2 is the 3Fe center. The ligation of these clusters by cysteine residues of the β subunit has been probed by a combination of site-directed mutagenesis, EPR spectroscopy, and redox potentiometry (309). The cysteines are positioned in the protein in four groups. Alternative models have been developed where the Fe-S clusters are arranged pairwise in centers 1 and 4 and in centers 3 and 2 or in a supercluster arrangement (309). In each case, a high-potential cluster and a low-potential cluster are juxtaposed. Cys→Ala substitutions of the putative ligands of clusters 2 and 3 result in the loss of the Fe-S centers and additionally also the Mo cofactor, showing that these centers play a strong structural role. Removal of cluster 1 or cluster 4 does not affect the remaining three clusters either spectroscopically or in their redox properties; therefore, clusters 1 and 4 are believed to be structurally less important.

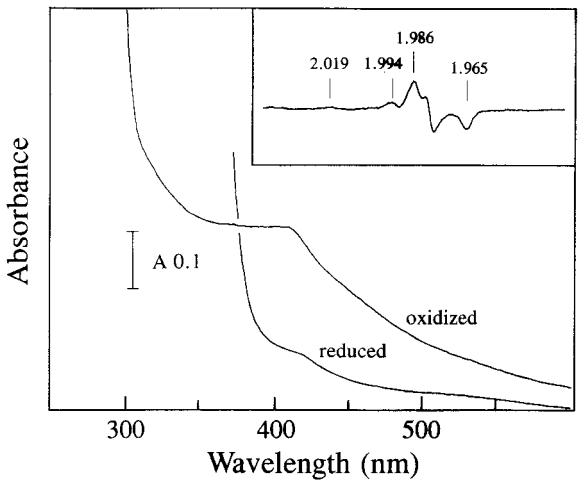

FIG. 6. Electronic absorption and EPR spectra of nitrate reductase from *P. stutzeri* ZoBell. The enzyme was in 20 mM phosphate buffer (pH 8.0). The oxidized form was as isolated; the reduced form was obtained by the addition of dithionite. Reproduced with permission from reference 84. (Insert) X-band EPR spectrum of the resting enzyme (pH 7.0). Microwave frequency, 9.236 GHz; modulation amplitude, 0.32 mT, modulation frequency, 100 kHz; microwave power, 1 mW; time constant, 200 ms; temperature, 78 K (407).

Nitrate reductases isolated in detergent carry a *b*-type cytochrome, as seen in the form of a third or γ subunit (19 to 23 kDa), encoded by the *narI* gene (Table 3). Such heterotrimeric enzymes exhibit the electronic spectrum and absorbance intensities of a diheme protein. The NarI function is ascribed to quinol oxidation and electron transport to the β subunit. The topology of the γ subunit of the *Paracoccus denitrificans* enzyme has been predicted by analogy to the NarI subunit from *E. coli* (192) as a transmembrane anchor that immobilizes the complex of the α and β subunits at the cytoplasmic side of the membrane (70). Heme ligation was proposed to be either by a single γ subunit or, based on known heme ligation of *b*-type cytochromes, by two equivalent subunits involving identical transmembrane helices (865). The minimal subunit stoichiometry is suggested to be heterotrimeric.

The αβ complex of the heat-released enzyme of *E. coli* has menaquinone-9 as a further constituent in a stoichiometry of one quinone molecule per protein dimer (103). The intramolecular electron transfer in nitrate reductase is thought to involve sequentially the two hemes of the γ subunit, the two Fe-S centers of the β subunit, and the molybdenum cofactor. The quinone is proposed to transfer electrons between the subunits or between the Fe-S clusters within the β subunit.

In a thus far singular case, the respiratory nitrate reductase of *E. coli* encoded by the *narGHJI* operon, enzyme A, is duplicated in the *narZYWV* operon encoding a similar enzyme Z (75% sequence identity between the α subunits) (80, 385). The subunits of the two enzymes are interchangeable and hence can form enzymatically active hybrid species such as $\alpha_A\beta_Z\gamma_Z$ or $\alpha_Z\beta_{A\gamma A}$ (81). The purified $\alpha_A\beta_Z$ enzyme is less stable and shows a somewhat decreased activity and looser membrane association. Preliminary evidence has been provided for two nitrate-reducing activities in *B. japonicum* whose underlying molecular entities are not clear (245). It should be noted that a nitrate-reducing activity can be associated in certain bacteria with another physiological activity, as is the case for the high-molecular-mass flavoheme protein nitrilotriacetate dehydrogenase (401).

Whereas the membrane-bound respiratory nitrate reductase is expressed only under anaerobic growth conditions, the periplasmic nitrate reductase is synthesized and active in the presence of oxygen (58, 762). Both enzymes are under nitrate control exerted via the sensor protein NarX or NarQ (see the section on regulation, below). In contrast to the membrane-bound nitrate reductase, the periplasmic enzyme does not reduce chlorate and *nap* mutants cannot be selected for by chlorate resistance (58). The physiological role of the periplasmic nitrate reductase is thought to consist of dissipating excess reducing power and providing nitrite for aerobic denitrification. In doing so, the enzyme may also function in the transition from aerobiosis to anaerobiosis. Since oxygen is believed to inhibit nitrate transport (335, 612), the periplasmic form of the enzyme could foster the transition to anaerobiosis.

Demonstration of the coexistence of the three types of nitrate reductases was achieved first with *R. eutropha* by physiological and mutational means (892). The periplasmic nitrate reductase of this organism was purified, and its structural genes were identified and sequenced (762). The enzyme was shown to be plasmid encoded and to consist of two subunits, NapA (93.3 kDa) and NapB (18.9 kDa) (Table 3). Both subunits are synthesized with signal peptides conforming to the periplasmic location of the mature gene products. The enzyme was later also isolated from a *narH* mutant of *Paracoccus denitrificans* GB17, and the analysis of the *nap* locus was extended to a *napEDABC* cluster (71).

The NapA subunit binds the molybdenum cofactor; a four-cysteine motif near the N terminus was proposed to bind a [4Fe-4S] cluster (101). NapA shows sequence relatedness to the respiratory nitrate reductase of *E. coli* and other molybdoenzymes such as the assimilatory nitrate reductase, formate dehydrogenase, and DMSO reductase of various organisms. Binding sites for Fe-S clusters and Mo were proposed from comparative sequence analysis (762). The sequence alignment of NapA with enzymes that bind MGD revealed two conserved regions that may be relevant for substrate specificity and contribute to the active site (71).

The small subunit NapB does not show similarities to other enzymes, but two potential heme C-binding sites are present in the sequence. Additional histidine residues are thought to effect a *bis*-histidine coordination of the hemes. The redox potentials of these hemes differ by about 100 mV (+80 and −15 mV) (72).

NapC belongs to a homologous family of tetraheme *c*-type cytochromes first reported as NirT of *P. stutzeri* (430). The putative role of NapC is in the electron transfer between a quinol and the periplasmic nitrate reductase. Homologs of *napC* exist in *R. eutropha* in the sequence reported adjacent to the *napAB* locus (762), in the *E. coli napFDAGHBC* locus linked to the genes for cytochrome *c* biogenesis (303), in the *Haemophilus influenzae* ORF HI0348 as part of a *nap* locus (250), and in *Rhodobacter sphaeroides* (679).

The functions of NapD and NapE for the NapAB complex are unknown, as are the functions of the *napGH* products of *E. coli*, which are predicted to be Fe-S proteins. Sequence-derived considerations place NapE in the membrane and NapD in the cytoplasm.

Periplasmic nitrate reductases have been studied in parallel in two strains of *Paracoccus denitrificans*. The activity and expression of periplasmic nitrate reductase in *P. denitrificans* PD1222 was demonstrated by physiological means (739) as well as by detecting a Mo(V) EPR signal in whole cells (738). The enzyme from *P. denitrificans* GB17 was subjected to biochemical and spectroscopic studies of its molybdenum (58, 61) and Fe-S (101) centers. Molybdenum coordination in the oxidized and reduced enzymes is different from that observed for the respiratory nitrate reductase. However, it resembles molybdenum coordination in assimilatory nitrate reductase, leading to the suggestion that these two soluble enzymes have similar Mo coordination spheres (62).

Molybdenum Cofactor

The hypothesis of an organic Mo cofactor as part of molybdenum-containing enzymes arose from the finding of pleiotropic effects on fungal assimilatory nitrate reductase and xanthine dehydrogenase. This concept gained momentum after interchangeability of low-molecular-mass compounds among molybdoenzymes had been demonstrated. A pivotal role in the experimental development of the field was played by the mutant *nit-1* of *Neurospora crassa*, of which a defective nitrate reductase could be reconstituted by cofactor donors. The principal findings until 1990 have been summarized (340). Molybdoenzymes carry the metal as part of a pterin cofactor, with the single exception of nitrogenase, where Mo is part of a polynuclear iron-sulfur cluster. Rajagopalan and coworkers have pioneered the elucidation of the cofactor molecule and inferred its structure from urothionine (667). The molecule, MPT (415), does not carry Mo, even though its name suggests otherwise, and the same cofactor molecule is found in tungsten-containing enzymes (131). It has also been pointed out that MPT does not fulfill the role of a true cofactor since it does not

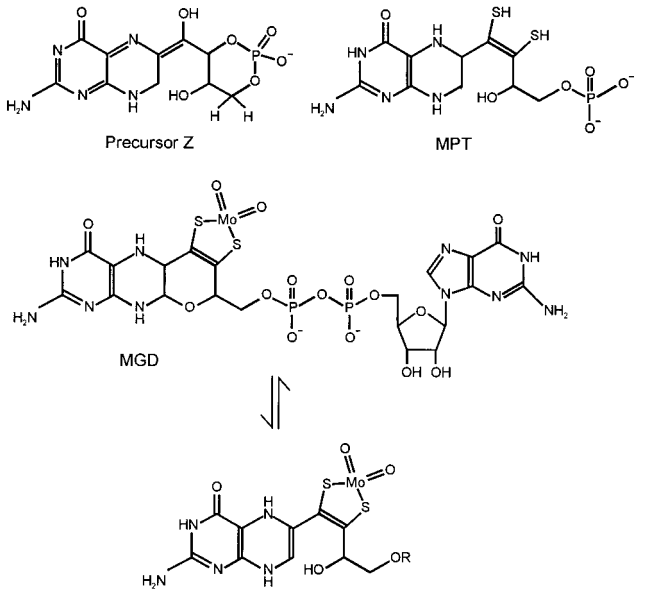

FIG. 7. Chemical structures of MPT, the MPT precursor Z, and the molybdenum form of MGD. The reversible formation of the pyran ring is indicated for MGD. The cofactor structures are shown in the tetrahydropterin form.

dissociate from the enzyme but, rather, is the prosthetic group of the respective molybdo- or tungstoenzymes (340).

The basic structure of the cofactor of the eukaryotic molybdoenzymes is a 6-alkyl pterin derivative with a phosphorylated, four-carbon side chain (Fig. 7) (470). In its active cofactor form, MPT is complexed with Mo by the sulfur atoms of the dithiolene configuration at the 6-alkyl side chain. The cofactor molecule of prokaryotic molybdoenzymes is composed of the pterin moiety and an additional group, lending a higher mass to the cofactor. The name "bactopterin" had been proposed initially for the modified bacterial Mo cofactor (570). Evidence for such a cofactor was provided for the respiratory nitrate reductase of *P. stutzeri* (480). The name "bactopterin" was not upheld when subsequent work with Mo enzymes revealed the existence of several cofactors distinguished by their nucleoside moiety.

DMSO reductase from the denitrifier *Rhodobacter sphaeroides* IL106 played a decisive role in unraveling the chemical nature of the bacterial cofactor as MGD (Fig. 7) (412). The cofactor structure was subsequently proven from the crystal structure of this enzyme (728). However, the first crystal structure of an MPT cofactor-containing protein, the tungstoenzyme aldehyde ferredoxin oxidreductase from *Pyrococcus furiosus* (131), unexpectedly revealed a three-membered ring system with a pyran ring and not an open configuration as thought previously. The pyran ring was also found in the cofactor of DMSO reductase. It is possible that ring closure is part of a redox process taking place on the enzyme-bound cofactor (222).

MGD is found in a variety of molybdoenzymes, among them formate dehydrogenases of both bacteria and archaea (400, 413), formylmethanofuran dehydrogenase of the methanogens (94, 439), and polysulfide reductase (400). Shortly after guanine was found, cytosine, adenine, or hypoxanthine was described for diverse prokaryotic Mo enzymes, of which some even seem to have more than one type of cofactor within the same protein molecule (94, 418, 667).

MGD is the cofactor of the respiratory nitrate reductases from the denitrifiers *P. carboxydoflava* and *P. stutzeri* (255, 256), the same cofactor as in the respiratory nitrate reductase from *E. coli* (416). Also, the periplasmic nitrate reductase of *Rhodobacter sphaeroides* is among the MGD-containing enzymes (667). A fluorescent compound, probably a pterin, has been extracted from the homologous enzyme of *Paracoccus denitrificans* GB17 (72), and since sequence comparison reveals similarity to other MGD-containing enzymes, NapA is considered to carry an MGD cofactor (69, 338).

In xanthine dehydrogenase of *P. aeruginosa*, only MPT, not the dinucleotide form, was found, which represents an exceptional case for bacterial molybdenum cofactors (414). However, MGD is present in cell extracts, as evidenced from the extraction of its oxidized, fluorescent derivative (427). Although the source of this MGD was not established and cells were not grown denitrifying, *P. aeruginosa* has the capability to synthesize MGD, and this cofactor is anticipated as part of its nitrate reductase. The evidence for MGD supports previous findings of restoration of nitrate reductase activity by mixing extracts of *P. aeruginosa* and a cofactor-deficient *mobB* strain of *E. coli* (713).

DMSO reductase of *R. sphaeroides* IL106 (a monomeric enzyme of 86.5 kDa with no other prosthetic group but the Mo cofactor) was shown to bind a single Mo atom via two MDG molecules (339). The crystal structure confirmed this stoichiometry and revealed that in the oxidized enzyme, two MDG molecules donate four sulfur ligands from the dithiolene groups to a six-coordinate Mo atom (728). The cofactors are extended and oriented in a mirrored and rotated opposite position. They differ by a 10° bend at the pyran ring and hence provide a slightly asymmetric environment. An oxo group and serine provide additional ligands for a distorted trigonal prismatic geometry around the Mo atom. The binding of Mo by MGD appears not to be uniform in MGD-containing enzymes. Although DMSO reductase of *Rhodobacter capsulatus* is a *bis*-MGD enzyme, only one MGD molecule complexes Mo, the other being too far removed from the metal site (733). In the pyrogallol transhydroxylase of the strict anaerobe *Pelobacter acidigallici*, the stoichiometry suggests a single Mo atom complexed between a heterodimer, with each subunit carrying an MGD molecule (673).

Based on sequence relatedness, dissimilatory nitrate reductases have been placed in the DMSO reductase family (69, 338). In respiratory nitrate reductase, three or four S ligands and a long Mo—O distance were detected by X-ray absorption to suggest a ligation similar to that of DMSO reductase (161, 275). On the other hand, the stoichiometry of MGD and Mo determined for nitrate reductase is 1:1, which implies that the additional S ligands are not from a second MGD molecule (255). Since it was initially not anticipated that one Mo atom can be complexed by two cofactor molecules in a molybdoenzyme, the stoichiometry of the cofactor and metal and the nature of the proximal Mo ligands of respiratory nitrate reductase warrant additional investigations. The role of the cofactor in the molybdoenzymes is thought to modulate the redox behavior of the metal and aid in the electron transfer from or to other redox centers without the pterin undergoing a redox process itself (338).

The biosynthesis of the Mo cofactor in *E. coli* has been intensively investigated and provided the principal picture shown in Fig. 8. It is hypothesized that the cofactor synthesis proceeds along the same basic pathway in other nitrate respirers including the denitrifying bacteria. Five distinct regions on the *E. coli* chromosome, *moa* (*chlA*), *moe* (*chlE*), *mob* (*chlB*), *mod* (*chlD*), and *mog* (*chlG*) participate in establishing a functional molybdoenzyme. They comprise loci necessary for MPT

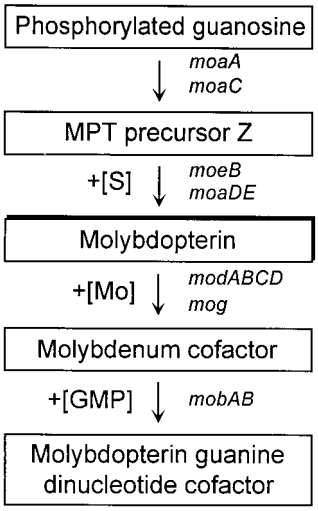

FIG. 8. Principal steps in the biosynthesis of the molybdenum cofactor. A phosphorylated guanosine species serves as the template upon which the biosynthetic enzymes act. These enzymes are indicated by their genetic acronyms; enzyme functions are discussed in the text. MPT is modified for bacterial molybdoenzymes by a nucleotide moiety; for the molybdenum cofactor of nitrate reductase, the guanylyl group is added to Mo-MPT. Relevant formulas are shown in Fig. 7.

biosynthesis and Mo uptake. The former *chl* designations, resulting from chlorate-resistant mutants with pleiotropic effects in molybdoenzyme activities, have been replaced by combining the *mo* symbol with the old *chl* allele (741).

MPT synthesis starts from a phosphorylated guanosine species (Fig. 8). Its synthesis resembles that of other pterins and riboflavin insofar as a guanosine derivative (usually GTP) serves as the template whose carbon atoms are incorporated into the pterin precursor. Whereas in the cyclohydrolase reaction for riboflavin and folate synthesis the C-8 atom of guanine is eliminated and does not appear in the products, the C-8 position is converted to the first C atom of the alkyl side chain of MPT. A novel route of biosynthesis is thus suggested for the Mo cofactor (923).

In the early steps of MPT synthesis, the products of the *moa* and *moe* loci are required (419, 688). *moaE* and *moeB* mutants accumulate the desulfo-MPT precursor Z (Fig. 7 and 8). In this precursor, the terminal phosphate is in a diester linkage to C-2′ and C-4′ of the side chain, forming a six-membered ring (922). Other than in riboflavin biosynthesis, where the ribose of GTP forms the ribityl side chain, both the ribose and the ring carbons of guanine are incorporated into the precursor Z of MPT. The *moa* locus of *E. coli* consists of a putative five-gene operon, *moaABCDE* (688). The MoaA protein (39 kDa) has been purified from *Arthrobacter nicotinovorans*. It is an Fe-S protein with a [3Fe-3S?] cluster whose coordinating cysteine residues are located in both the N- and the C-terminal regions. The candidate ligands, Cys32, Cys39, Cys277, and Cys294, have been identified by site-directed mutagenesis (564). The protein is suggested to act as an oxidoreductase or as the sulfur donor to MPT synthase. The N-terminal region of MoaA with the cysteine cluster shows some similarity to the NifB protein, which is involved in the FeMo cofactor synthesis of nitrogenase (563, 688). For a discussion of the similarity of MoaA to the NirJ and Pqq proteins, see the section on heme D_1 biosynthesis, below.

The products of the *moaD* and *moaE* genes (8.8 and 16.9 kDa, respectively) are the two subunits of the MPT synthase (formerly the converting factor). The protein has been purified, but since the subunits are not tightly bound, the degree of oligomerization is not known (650). The function of the synthase is the introduction of the dithiolene sulfurs into precursor Z (Fig. 8). The sulfur is provided from an unknown sulfur source to the MoeB protein, from where it is transferred to the small subunit of the synthase. The presence of reactive sulfur is traceable to MoaE from its reactivity with iodoacetamide.

In the following steps of cofactor synthesis, the products of the *mod* and *mog* loci are involved. The *mod* (*chlD*) locus encodes components used for molybdenum uptake (281) and is regulated by the availability of molybdate in the medium (580). Molybdenum transport and processing systems must be intimately associated with the biosynthesis of the denitrification apparatus to provide the metal to the respiratory and periplasmic nitrate reductases. The *narD* locus of *P. aeruginosa* may correspond to *chlD*, since a mutation is rescued by exogenous molybdate (867). It is likely that the molybdenum uptake process in diazotrophic denitrifiers is shared for the two different types of molybdenum cofactors before more specific functions take over to yield either one. Transport studies have been undertaken with *Bradyrhizobium japonicum* (529) and *Azospirillum brasilense* (141), which are both diazotrophic denitrifiers. Bacteroids of *B. japonicum* have a high-affinity uptake system (K_m, 0.1 μM) that is anaerobically more active. Molybdate uptake is thought to occur by proton symport since the system is sensitive to uncouplers and ionophores. Molybdate uptake is competitively inhibited by tungstate. Radioactive [^{99}Mo]molybdate is very strongly bound and appears to be immediately channeled to intermediate binding proteins or to the respective target enzyme. A 100-fold excess of unlabeled molybdate is not able to exchange Mo even after only 1 min of uptake of the latter (529).

The *modABCD* operons encoding molybdenum transporters have been unraveled in *Azotobacter vinelandii* (525), *Rhodobacter capsulatus* (890), and *E. coli* (551, 670). Homologous genes were also detected in the genome of *H. influenzae* (250). The uptake system of *E. coli* is an energy-dependent high-affinity system with a K_m of 25 to 27 nM. It depends on a periplasmic binding protein with a low dissociation constant for molybdate (520). *mod* mutants are affected in molybdate uptake (330, 520, 701). A high exogenous molybdate concentration can rescue a *mod* mutation, with molybdate uptake then proceeding via sulfate or selenate transporters with a lower efficiency (701).

A partial sequence of the transport operon, consisting of *modC* and the upstream and downstream flanking genes, revealed that *modC* encodes a 39-kDa protein with similarity to the ATPases of the high-affinity ABC transporters (410). Members of the family of ABC transporters export and import a wide range of substances across membranes (336). Their architecture consists of variations around a basic model of a periplasmic substrate-binding protein, the membrane-bound transporter, and a cytoplasmic ATPase. ABC transporters are also relevant to other aspects of denitrification.

ModA (*chlX*) is the periplasmic binding protein (26.4 kDa) for the molybdate transporter. It has been overexpressed and purified, which allowed its biochemical characterization. On binding of molybdate, ModA undergoes alterations in its electrophoretic mobility and exhibits a new absorption maximum at 287 nm. Purified ModA binds molybdate with a K_d of 3 μM (671); a previous estimate obtained with unfractionated periplasmic material gave a value of only 9 nM (520). It is not clear whether the discrepancy of the determinations is due to the differences in materials tested. Cells grown in medium containing <10 nM molybdate still synthesize appreciable lev-

els of cofactor and nitrate reductase, which implies a high affinity of the transporter to molybdate (736). *modB* (*chlJ*) encodes a hydrophobic transmembrane protein, considered to be the transmembrane carrier for molybdate. The role of *modD* is still unclear; at least the gene does not appear to be essential.

The *mod* operon is under the control of the ModE (= ModR), repressor whose gene is located immediately upstream of and transcribed oppositely to *modA* (304, 887). In the presence of molybdate, ModE binds preferentially to a 9-bp palindromic recognition sequence, CGTTATATA-N_{4-12}-TATATAACG, at the *modA* promoter from −18 to +10 relative to the start of transcription (14, 557). The protein acts as a dimer and binds two molecules of molybdate with a K_d of 0.8 μM. Homologs of *modE*, but no corresponding regulatory regions, are present in the *mod* region of the diazotrophs *Azotobacter vinelandii* and *Rhodobacter capsulatus* B10. Instead of a single *modE* homolog, the region upstream of *modA* of *R. capsulatus* harbors the divergently transcribed *mopA* (34% identity to *E. coli* ModE) and *mopB* genes, which are both suggested to encode pterin-binding proteins (890). It is not known whether *R. capsulatus* B10 reflects the situation of denitrifying members of the genus *Rhodobacter* and other nonphotosynthetic denitrifiers, of which none have yet been investigated for their molybdate uptake system.

The chelatase for Mo insertion into the cofactor is thought to be encoded by the *mog* locus (Fig. 8). If MPT is not loaded with the metal or if the cofactor is not inserted into an acceptor enzyme, it is rapidly degraded, such as in *mog* and *mod* mutants (426).

The *mob* genes are involved in a late step of MGD synthesis (416). The *mob* locus comprises *mobA* and *mobB*, whose expression is constitutive at a low level (384). The inactive soluble precursor of nitrate reductase from *mob* mutants already contains MPT. The in vitro activation of purified *mob* nitrate reductase depends on the *mobA* product, MgGTP, and a further activity ascribed to a factor X (712). A *mobB* mutant has no recognized phenotype, but overexpression of *mobB* enhances factor X activity (635). The gene-deduced protein has a putative GTP-binding site to suggest a role for MobB in MGD synthesis, yet its function appears to be redundant, since a *mobB* deletion mutant continues to synthesizes active nitrate reductase, trimethylamine *N*-oxide reductase, and formate dehydrogenase. All three enzymes contain MGD (635). The protein has been purified from an overexpressing strain. It is a dimeric molecule (subunit mass, 19.5 kDa) that binds ≈0.8 mol of GTP/mol with a K_d of 2 μM (229). Its properties are compatible with a role of binding the guanine nucleotide that becomes incorporated into MGD. *mobA* encodes the FA protein (association factor), which has also been purified to homogeneity. The factor is a low-molecular-mass monomeric protein (21.6 kDa) and activates inactive molybdoenzymes of *mob* mutants (229, 636). There is a limited sequence similarity of 80 C-terminal residues of MobA to the regions of NarG and NarZ of *E. coli*, which are suggested to bind the cofactor. MobA may therefore interact with the cofactor and attach the nucleotide moiety (416).

Cofactor synthesis must incorporate an element for discrimination among different types of Mo cofactors present in denitrifying bacteria. MGD is the cofactor of *Hydrogenophaga* (formerly *Pseudomonas*) *pseudoflava* nitrate reductase, but MPT cytosine dinucleotide is the cofactor of the CO dehydrogenase of the same organism (569). MPT and MGD coexist in *P. aeruginosa* (427) and have to be provided for xanthine dehydrogenase and nitrate reductase, respectively. Specificity could be part of the insertion process or could reside in different *mob*-encoded proteins. The sequence of the late events has not been explicitly established. Charging of MPT with molybdenum, insertion into a target protein, and addition of the nucleotide moiety can be deduced from the phenotypes of *mog*, *mod*, and *mob* mutants. Recently it has been found that NarJ, encoded within the structural operon for nitrate reductase, is part of factor X activity and renders the maturation process of the *mob*-encoded proteins specific for nitrate reductase (635).

Transport of Nitrate and Nitrite

The catalytic site of nitrate reductase is oriented toward the cytoplasm and generates nitrite at the inner face of the membrane (411, 424, 471). In contrast, nitrite reductases are periplasmic enzymes in gram-negative bacteria. With the sites of reduction of the two oxyanions at opposite faces of the membrane, transport systems for nitrate and nitrite are required. As yet, the prokaryotic nitrate/nitrite transporter has not been identified at the molecular level in any denitrifying bacterium. An antiport system is thought to control the movements of nitrate and nitrite across the membrane during nitrate respiration (93, 180). Since no change in net charge affecting the membrane potential is associated with the counter movement of these anions, an antiporter is energetically the most favorable proposition. A nitrate/proton symport, driven by the energized membrane, has been proposed, both as the principal uptake process and to initiate nitrate uptake prior to the functioning of the antiport system (93, 473). Recent data confirm an energy requirement for nitrate uptake that is sensitive to protonophores and may favor a symport mechanism (482, 706, 917). However, there are also contradictory findings (334, 639), and a passive nitrate-specific pore has been postulated to account for the lack of energy requirement and missing unequivocal evidence for an antiporter. Perhaps the most efficient nitrate uptake system of a denitrifier has been found in the vacuolated sulfide- and elemental sulfur-oxidizing bacterium *Thioploca* sp., where nitrate is concentrated from 25 μM in seawater to 0.5 M inside the cell (253).

Transport systems for nitrate and nitrite are better known outside the denitrifying bacteria. In *E. coli*, a nitrite exporter (the *narK* product) is encoded as part of the *narLXKGHJI* gene cluster for respiratory nitrate reduction (80, 611). Homologs of NarK are anticipated in denitrifiers, since they are also found in the gram-positive nitrate respirers *Bacillus subtilis* (168) and *Staphylococcus carnosus* (237). The 12-span membrane-bound NarK protein of *E. coli* was initially thought to be a nitrate transporter since deletion of *narK* affected nitrate uptake. It was later believed to constitute the NO_3^-/NO_2^- antiporter (193). A more recent investigation making use of membrane vesicles (rather than intact cells) and of sensitive techniques (the use of [^{13}N]nitrate and quenching of a fluorescent dye by NO_2^-) suggests that the physiological role of NarK is that of a nitrite exporter and leaves the search for the proper nitrate uptake system open again (706). Sequence analysis indicates that NarK belongs to the major facilitator superfamily of transporters. This family has a broad substrate spectrum that includes H^+, sugars, and antibiotics; its members share 12 membrane-spanning segments and several sequence motifs (535, 837). NarK is thus related to the nitrate transporter and bispecific nitrate/nitrite transporter of eukaryotic microorganisms of this family (265, 838, 846).

A different type of nitrate transporter which belongs to the ABC family of transporters has been identified in cyanobacteria and heterotrophic nitrate-assimilating bacteria and has

TABLE 4. Distribution of the two types of nitrite reductases among denitrifying prokaryotes

Cytochrome cd_1 nitrite reductase	Reference(s)	Cu-containing nitrite reductase	Reference
Ralstonia eutropha	711	"*Achromobacter cycloclastes*"	391
Alcaligenes faecalis IAM 1015	389	*Alcaligenes faecalis* S-6	433
Azospirillum brasilense SP7	179	*Alcaligenes xylosoxidans* subsp. *xylosoxidans*	393
Magnetospirillum magnetotacticum	617, 934	*Bacillus halodenitrificans*	198
Paracoccus denitrificans	585, 607, 822	*Haloferax denitrificans*	380
Halomonas halodenitrificans	532	*Nitrosomonas europaea*	579
Pseudomonas aeruginosa	930	*Pseudomonas aureofaciens*	977
Pseudomonas nautica	75	*Rhodobacter sphaeroides*	720
Pseudomonas stutzeri	517, 896, 982		
Roseobacter denitrificans	212		
Thiobacillus denitrificans	375		

been partially characterized biochemically (515, 622, 637). This transport system is repressed and immediately inhibited by ammonia (76). The cyanobacterial and enterobacterial uptake systems are homologous. NrtA (homologous to NasF) represents the presumed periplasmic binding protein for nitrate, which is membrane inserted in *Synechococcus* sp. strain PCC 7942. NrtB (homologous to NasE) is the membrane-bound transporter, and NrtD (homologous to NasD) is the cytoplasmic ATPase. The synechococcal transporter has an additional cytoplasmic component not found in *Klebsiella pneumoniae* (622). Besides nitrate, the Nrt system also transports nitrite (526).

RESPIRATION OF NITRITE

Biological Redundancy in Nitrite Reductases

For the reduction of nitrite, one finds in denitrifying bacteria, although never within the same cell, two entirely different enzymes in terms of structure and the prosthetic metal. About three-quarters of strains collected worldwide, with a prevalence of pseudomonads among them, have the tetraheme protein cytochrome cd_1 as the respiratory nitrite reductase (266). The same reaction is catalyzed by a CuNIR in a greater variety of physiological groups and bacteria from different habitats. In 23 strains from culture collections, a slight predominance of CuNIR was found, whereas the numerically dominant isolates and denitrifiers from aerobic soil enrichments have mostly cytochrome cd_1 (158). Whether the numerical prevalence of strains with cytochrome cd_1 reflects a real dominance over CuNIR or only a preferential isolation of such denitrifiers is unknown. Methods for assessing the full denitrification potential of soil and water samples are still under development. Improvements of DNA probes recognizing denitrification genes and the definition of diagnostic sequences is necessary to reach meaningful data for natural, noncultured assemblages. Eventually, this should provide a detailed picture of the true denitrification potential and its underlying bacterial diversity of natural samples.

Table 4 lists the sources of the two types of nitrite reductases for which an unequivocal identification by partial purification and spectral evidence has been reported. Within the *Proteobacteria*, neither CuNIR nor cytochrome cd_1 is found in exclusive association with a particular subclass, but both types are found in each one of the alpha, beta, and gamma subclasses (965). The genetic information for either nitrite-reducing system coexists occasionally at the genus level but, as far as we currently know, not at the species level (Table 4). The distribution of the two types of enzymes has been studied by DNA-DNA hybridization with the structural genes *nirS* (774) and *nirU* (= *nirK*) (941) and by cross-reaction with an antiserum against CuNIR (158). In most instances, the immunochemical data agree quite well with those from DNA hybridization.

Since cytochrome cd_1 is not inhibited by DDC, a preliminary diagnosis of whether a new isolate possesses a CuNIR can be made by inhibiting nitrite reduction in cell extract with this chelator (743). However, azurin and pseudoazurin are among the electron donors for cytochrome cd_1 and are also affected by DDC. A further complication is the effect of this chelator on electron transfer components of the respiratory chain (481). The use of size-fractionated soluble material to ensure the removal of the low-molecular-weight Cu proteins and membrane vesicles and the use of an artificial electron donor system make this test less prone to error. Thus, in the case of *Paracoccus denitrificans* GB17, the initial conclusion of a CuNIR based on the use of DDC did not survive closer scrutiny (585). Preliminary evidence for a CuNIR from inhibition by DDC has been reported for *Bacillus firmus* (751), *B. stearothermophilus* (346), *Bradyrhizobium japonicum* (158), *Nitrobacter vulgaris* (6), and *Rhodopseudomonas palustris* (658).

The interchangeability of a CuNIR with cytochrome cd_1 has been studied by using *nirK* from *P. aureofaciens* and *nirS* from *P. stutzeri* (282). CuNIR was found to be active in a mutationally cytochrome cd_1-free background of *P. stutzeri* and restored the interrupted denitrification activity. In contrast, expression of *nirS* in *P. aureofaciens* resulted in an inactive enzyme species because the requirements for heme D_1 biosynthesis are not met by the host organism. The capability of *P. stutzeri* to express *nirK* functionally in vivo is remarkable in light of the electron donor specificity of the enzyme. NirK activity of *P. aureofaciens* is dependent on its indigenous azurin (977), but a cupredoxin is not present in *P. stutzeri*. Thus, electron donation to heterologous NirK must involve a foreign electron carrier, in all likelihood a *c*-type cytochrome taking advantage of a low recognition specificity of the reductase. The underlying phenomenon of the interchangeability of electron donors for nitrite reductases is discussed below in more detail in the section on electron donation.

Cu-Containing Nitrite Reductase

Enzyme properties. On the basis of their optical and EPR properties, Cu(II) centers in proteins are assigned to one of three classes, type 1 (blue) Cu, type 2 (nonblue) Cu, and type 3 (binuclear and EPR-inactive) Cu (530). The first nitrite reductase with Cu as prosthetic metal was discovered in Mori's laboratory (393). The basic properties established then were evidence for Cu from chemical analysis, restoration of catalytic activity of a metal-depleted enzyme by Cu, and inhibition of the enzyme by DDC. The enzyme, although not difficult to

purify and in spite of its importance in the N cycle, did not enter the mainstream of Cu-protein research for many years. This has changed dramatically since. The crystal structure of CuNIR, the first of any denitrification enzyme (284), was a breakthrough and motivated subsequent studies on the reaction mechanism, electron transfer, and the interaction of electron carriers with their cognate reductases.

A survey of the enzymes purified from various sources reveals apparent differences in absorbance properties (blue versus green proteins), molecular masses, the subunit composition of the holoenzymes, and catalytic activities (Table 5). A seeming heterogeneity among the CuNIR species had been a general feature until recently and contrasted with the more uniform family of cytochrome cd_1 nitrite reductases. Evidence from three X-ray structures and four gene-derived amino acid sequences clearly reveal CuNIR species to be members of the same protein family. Crystal structures and X-ray scattering have also firmly established that CuNIR species are trimeric enzymes (208, 284, 301, 323, 488).

The size of subunits determined by SDS-electrophoresis is close to 40 kDa in each case, independent of the suggested quaternary structure (Table 5). Some CuNIR species are proteolytically sensitive. Three peptides were observed in *Bacillus halodenitrificans* (198). The *Rhodobacter sphaeroides* IL106 enzyme yields a single subunit or two different species, depending on the purification procedure (575). As far as sequences are known, all nitrite reductases have a related primary structure of a single-type subunit with a positional amino acid identity of 61 to 81%. The first amino acid sequence was determined from overlapping proteolytic peptides of CuNIR from "*A. cycloclastes*" (244). Homologous structures were deduced from the nitrite reductase genes of strain G-179 of *Pseudomonas* sp. (941), *Alcaligenes faecalis* S-6 (608) and *P. aureofaciens* (282). The most distant proteins obtained from *P. aureofaciens* and "*A. cycloclastes*" still have 61% sequence identity. The Cu-coordinating peptides are identical in the four enzymes with respect to their general location in the protein and the surrounding regions of conserved amino acids (Fig. 9).

The presence of type 1 and type 2 Cu is manifest in the small and large hyperfine splitting in the EPR spectrum of nearly all nitrite reductases (Fig. 10; Table 5). The analytically determined amount of Cu, however, is smaller than the expected number of six Cu atoms (Table 5). Often, only half of the theoretically amount has been detected by chemical means. Close to five Cu atoms per trimer are present in the "*A. cycloclastes*" enzyme when the reported number is corrected for a mass of 109.4 kDa (516). A metal-depleted enzyme was reconstituted with Cu(II), and 5.3 to 5.6 Cu atoms were found by colorimetry and EPR spectroscopy (514). A lower than stoichiometric content of Cu may be due to the possibility that part of the Cu sites is occupied by Zn (1).

The hydrodynamic properties of nitrite reductase indicate rapid dissociation-reassociation equilibria (284, 301). With a monomeric species forming part of these equilibria, loss of Cu may result during enzyme purification and explain why in two instances a protein with only the type 1 Cu in place has been isolated (539, 977). A 20-fold loss of enzyme activity is observed on cell breakage with the *Alcaligenes xylosoxidans* enzyme, which is counteracted by incubating the crude extract with 1 mM $CuSO_4$ (1). Spontaneous depletion of the type 2 Cu occurs on storing the enzyme from "*A. cycloclastes*" for 2 to 3 days at 4°C before ammonium sulfate precipitation, an effect that has been used to demonstrate by metal reconstitution the requirement of type 2 Cu for CuNIR catalysis (514).

The *Alcaligenes xylosoxidans* enzyme is activated by a factor of up to 40 by freeze-thawing (538). Slow freezing may cause a rearrangement of the Cu centers and stimulate enzyme activity by filling part of the type 2 center. Unfortunately, the EPR characteristics of the material before and after treatment were not reported. Repeated chromatographic passage over hydroxylapatite and TSK column material produces type 2 Cu signals in the EPR spectrum of the *P. aureofaciens* enzyme that were absent in the original material (977). Since type 1 Cu is usually very tightly bound and is removable only under drastic conditions, the source of the filling of the type 2 Cu site is unknown and warrants further scrutiny.

All CuNIR species exhibit electronic absorbance around similar wavelengths (Fig. 11 and Table 5). Usually there is a prominent set of absorbances in the visible region around 460, 590, and 700 to 750 nm, with additional shoulders at shorter and longer wavelengths. CuNIR from *Alcaligenes xylosoxidans* and *P. aureofaciens* are blue enzymes and show very little absorption in the 450-nm region, whereas the other enzymes are green. The type 1 site is the chromophoric center since the type 2-depleted enzyme from "*A. cycloclastes*" remains green (4). The set of ligands of the type 1 center in the green reductases, $(His)_2$-Cys-Met, is the same as in azurin or other cupredoxins. In the green reductases, the absorption at 590 nm is reduced and that around 460 nm is greatly enhanced. This difference reflects the degree of distortion of the tetrahedral, type 1 Cu-binding site (316, 499). Methionine and cysteine are slightly moved with respect to their position in a classical type 1 site. The Cu-S distance is somewhat shorter in the green than in the blue nitrite reductase (4). Green azurins can be engineered by introducing rhombic distortion into type 1 Cu sites by a strong axial ligand, for instance a Met121His substition (reviewed in reference 118).

Structure. The first three-dimensional structure analysis of a CuNIR was achieved with the enzyme from "*A. cycloclastes*." A detailed description of the refined structure, of crystal forms at various pH values, and of the substrate-bound form was provided recently by Adman et al. (4). In the crystal, the enzyme subunits are tightly associated around a threefold axis to form a trimer around a central channel of 5 to 6 Å diameter (Fig. 12).

Each subunit of the trimer comprises two domains. Their polypeptide fold is a "Greek key" β barrel, similar to the type 1 blue proteins or cupredoxins (3, 4). The barrels are stacked onto each other, and two extended loops interact between domains I and II. Only nine hydrogen bonds exist between the two domains. Contributing to the stabilization of the molecule are surface interactions that comprise 28% of the monomer surface on trimer formation. The type 1 Cu site is formed from adjacent residues of domain I. The residues His95, Cys136, His145, and Met150 form a flattened tetrahedron similar to the Cu center in pseudoazurin (284). Type 2 Cu is bound by three histidine ligands (His100, His135, and His306) provided from domain II. However, His306 comes from the adjacent subunit; i.e., Cu is bound at the interface of two subunits (Fig. 12). The type 2 Cu is not required for the conformational stability of the trimer. Solution X-ray scattering was found to be unchanged for the type 2-depleted enzyme (796).

Together with a water molecule, the ligands of the type 2 Cu form an unusual pseudotetrahedral geometry. A fourth histidine residue, His255, is nearby but is not a ligand in the crystal form obtained at pH 5.2. All histidines remain oriented in the same place on removal of the type 2 Cu. The distance to be bridged by electron transfer between the type 1 and type 2 Cu sites within the monomer is 13 Å.

A second crystal structure of a green enzyme was obtained with the nitrite reductase from *Alcaligenes faecalis* (488). It confirms the Cu-binding residues and the trimeric nature of

TABLE 5. Properties of copper-containing nitrite reductases (EC 1.7.2.1)

Property	Value for:						
	Pseudomonas aureofaciens	*Alcaligenes xylosoxidans*	"*Achromobacter cycloclastes*"	*Alcaligenes faecalis* S-6	*Rhodobacter sphaeroides*	*Bacillus halodenitrificans*	*Nitrosomonas europaea*
Mol mass (kDa)	85[a]	103[b]	105[b]	100.4–119[b]	80[a]	82[a]	127.5[a]
No. and mass (kDa) of subunits	3,[c] 36.9[d]	3,[e] 36.5[d]	3,[e] 36.5[d,e]	3,[e] 37[d]	37.5, 39.5[f]	40[f]	3, 40.1[f]
pI	6.05	8.4		4.5	5.2(α), 5.0(α′)		4.63
Visible absorbance (nm) (protein as isolated)	474, 595, 780	460, 593, 770	400, 464, 590, 700	400, 457, 587, 700	463, 585, 740, 840	454, 595, 710, 800	607
ε (mM^{-1} cm^{-1}), [λ_{max}, nm]	7.0 [595]	6.3 [593]	6.3 [464][h]	6.98 [457]	4.86 [585]	4.94 [595]	6.0 [607]
No. of Cu atoms/holoenzyme	1.96	3.5 ± 0.8	4.6[g]	4.5	2	1.56	3.7
EPR parameters							
Type 1 Cu, $g_{\parallel}$	2.218	2.208	2.195	2.19	2.173	2.23	2.25
Type 1 Cu, $A_{\parallel}$ (mT)	6.36	6.3	7.3		7.8	4.9	6.8
Type 2 Cu, $g_{\parallel}$	Present	2.298	2.262	2.30	2.324	2.32	2.26
Type 2 Cu, $A_{\parallel}$ (mT)	10	14.2	17.5		16.3	12.9	17.0
Electron donors	Azurin, PMS-asc, MV-$S_2O_4^{2-}$	Cytochrome c_{553}, PMS-asc, MV-$S_2O_4^{2-}$	Pseudoazurin, cytochrome *c*?	Pseudoazurin, PMS-asc, MV-$S_2O_4^{2-}$	Cytochrome c_2, PMS-asc, BV-$S_2O_4^{2-}$	PMS/asc	Cytochrome c_{552}
Activities ($\mu mol \cdot min^{-1} \cdot mg^{-1}$)							
$NO_2^- \rightarrow NO$	1		280	380 (MV)	40 (BV)	90	12.5[i]
$NO_2^- \rightarrow NH_3$		240 (MV)					
$NH_2OH + NO_2^- \rightarrow N_2O$	+		+			1.4	
Oxidase	−	+	+		+	−	+
K_m for NO_2^- (μM)		230	500	740 (MV)	30–50 (BV)		
Inhibitors	DDC, CN^-		DDC, CN^-, CO	DDC, CN^-	DDC, CN^-, CO	DDC, CN^-, EDTA	
Reference(s)	282, 977	1, 208, 538, 539	244, 284, 390, 391, 516	432, 433, 488	575, 720	198	206, 579

[a] By gel filtration.
[b] By sedimentation equilibrium.
[c] Trimeric structure derived from X-ray scattering.
[d] Sequence-derived value.
[e] Trimeric structure derived from crystal structure.
[f] By SDS-gel electrophoresis.
[g] Value from reference 516 corrected for M_r 109,400.
[h] Value from reference 390 corrected for M_r 109,400.
[i] Assayed as oxidation of cytochrome c_{552}; the reaction product was not identified (206).

```
                                 1    2                                  21         1    1
P. aureofaciens      105 NPATNSMPHNVDFHAATGALGGAGLTQVVPGQEVVLRFKADRSGTFVYHCAPQG-MVPWHVVSGMNGALMVLP 176
Pseudomonas sp. G179 126 NPDTNELQHNIDFHSATGALGGGALTVVNPGDTAVLRFKATKAGVFVYHCAPAG-MVPWHVTSGMNGAIMVLP 197
A. cycloclastes      125 NPDTNTLLHNIDFHAATGALGGGALTQVNPGEETTLRFKATKPGVFVYHCAPEG-MVPWHVTSGMNGAIMVLP 196
R. hedysari          124 NPETNTLQHNIDFHSATGALGGGALTVVNPGESAVLRFKATKAGVFVYHCAPPG-MVPWHVTSGMNGAIMVLP 195
R. sphaeroides       118 NPPENTMPHNIDFHAATGALGGGGLTLINPGEKVVLRFKATRAGAFVYHCAPGGPMIPWHVVSGMAGCIMVLP 190
A. faecalis          123 NPETNTLMHNIDFHAATGALGGGGLTEINPGEKTILRFKATKPGVFVYHCAPPG-MVPWHVVSGMNGAIMVLP 194
                         **  * ° **°***°*******  ** ° **    ***** ° * ******* * *°**** *** *°°****

                                                          2
P. aureofaciens      291 PPQRNMETWFIPGGSAVAALYTFKQPGTYVYLSHNLIEAMELGALAQIKVEGQWDDDLMTQVKAPGPIVEPKQ 363
Pseudomonas sp. G179 312 PPELDQETWFIPGGAAGAAYYTFQQPGIYAYVNHNLIEAFELGAAGHFKVTGDWNDDLMTAVVSPTSG      379
A. cycloclastes      311 PPDLDQETWLIPGGTAGAAFYTFRQPGVYAYVNHNLIEAFELGAAGHFKVTGEWNDDLMTSVVKPASM      378
R. hedysari          310 VPDRDQETWFIPGPTRGAAYYTFEQPGIYAYVNHNLIEAFELGAAAHFKVTGDWNDDLMTTVRSPSGS      377
R. sphaeroides       305 APERDLETWFIRGGTAGAALYKFLQPGVYAYVNHNLIEAVHKGATASVLVEGEWDNDLMEQVWPPVGLTG    374
A. faecalis          309 PPDVDQETWFIPGGAAGAAFYTFQQPGIYAYVNHNLIEAFELGAAAHFKVTGEWNDDLMTSVLAPSGT      376
                          *    *** * * °  ** * * *** * *° ******   **     * * *  ***  *  *
```

FIG. 9. Sequence alignment of the metal-coordinating regions of Cu-containing nitrite reductases. The Cu-binding residues are shown in negative print; 1, ligands to the type 1 Cu; 2, ligands to the type 2 Cu. Asterisks and small circles denote identical and conserved residues, respectively; the C-terminal residues in the lower block are shown in boldface type. For sources of gene-derived sequences, see Table 2.

the enzyme and the other principal conclusions drawn from the "*A. cycloclastes*" enzyme. The structure of the blue enzyme from *Alcaligenes xylosoxidans* was recently reported and confirms that there are only subtle differences in the bond length of the Met-sulfur to Cu and angles with respect to those in the green form of CuNIR (208).

Mechanistic aspects. CuNIR forms in vitro NO from nitrite with PMS-asc as the electron donor system (Table 5). With the low-potential electron donor system of MV and dithionite, N_2O and ammonia formation have been reported also. The *Alcaligenes faecalis* S-6 and *Rhodobacter sphaeroides* IL106 enzymes produce N_2O with MV and dithionite. A nonenzymatic further reduction of NO to N_2O takes place in this case. With PMS-asc as the reducing system the *Alcaligenes faecalis* enzyme generates NO and about 6% of N_2O (433); that from "*A. cycloclastes*" generates about 3% N_2O besides NO (396). Several nitrite reductases were shown to form N_2O from nitrite with hydroxylamine, and some also reduce O_2 (Table 5).

The CuNIR of *Alcaligenes xylosoxidans*, in either a cell extract or the soluble cell fraction, forms ammonia from nitrite with MV plus dithionite. The reaction is believed to comprise the conversion of NO to NH_2OH as a nonenzymatic step with cytochrome c' as a hydroxylamine reductase (538). Ammonia formation from nitrite with MV plus dithionite and NO formation from nitrite with PMS plus asc have also been confirmed to occur with the purified enzyme (1). A different mechanism is required, since this excludes a catalytic contribution from cytochrome c'. The formulation of the active-site chemistry will have to account for this enzyme versatility.

The type 2 center is the substrate-binding site of nitrite reductase. Type 2-depleted enzyme is nearly inactive, but its activity increases markedly on reconstitution of the type 2 center (514). The spontaneous loss of Cu during the preparation of the *Alcaligenes xylosoxidans* enzyme makes it feasible to compare species having only the type 1 Cu center occupied with species having both Cu centers filled. The EPR parameters of the type 2 Cu change on addition of nitrite from $g_{\parallel}$ 2.355 to 2.290; $A_{\parallel}$ increases by 2 mT. In contrast, there is no change in $g_{\parallel}$ or the A tensor for type 1 Cu (366). The ^1H and ^{14}N electron nuclear double resonance (ENDOR) spectra of the type 2 Cu recorded at $g_{\parallel}$ also change on addition of nitrite, whereas the equivalent spectra of the type 1 Cu are perturbed only to an insignificant extent. Both observations imply a rearrangement of ligands at the type 2 center by the substrate nitrite. The extended X-ray absorption fine structure of the type 2-depleted enzyme closely resembles that for cupredoxins and remains unperturbed in the presence of nitrite, whereas that of the holoenzyme changes on nitrite binding (796). General features for CuNIR thus comprise the trimeric structure,

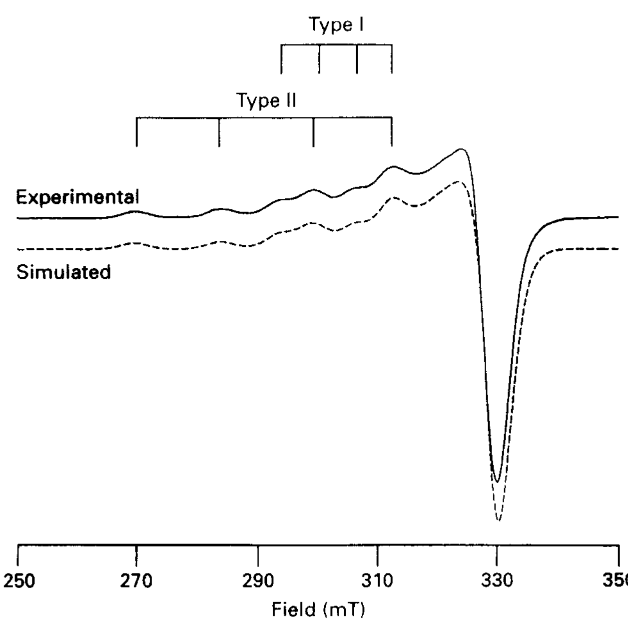

FIG. 10. X-band EPR spectrum of Cu-containing nitrite reductase from *Alcaligenes xylosoxidans* showing the hyperfine splittings of type 1 and type 2 Cu. Solid line, enzyme sample, 62 mg/ml, recorded at 120 K, 10-mW microwave power, 9.37 GHz, and 0.53-mT field modulation at 100 kHz. Dotted line, simulated spectrum with the parameters listed in Table 3 of reference 1 and a contribution of about 60% type 1 and 40% type 2 Cu to the spectrum. Reproduced with permission from reference 1.

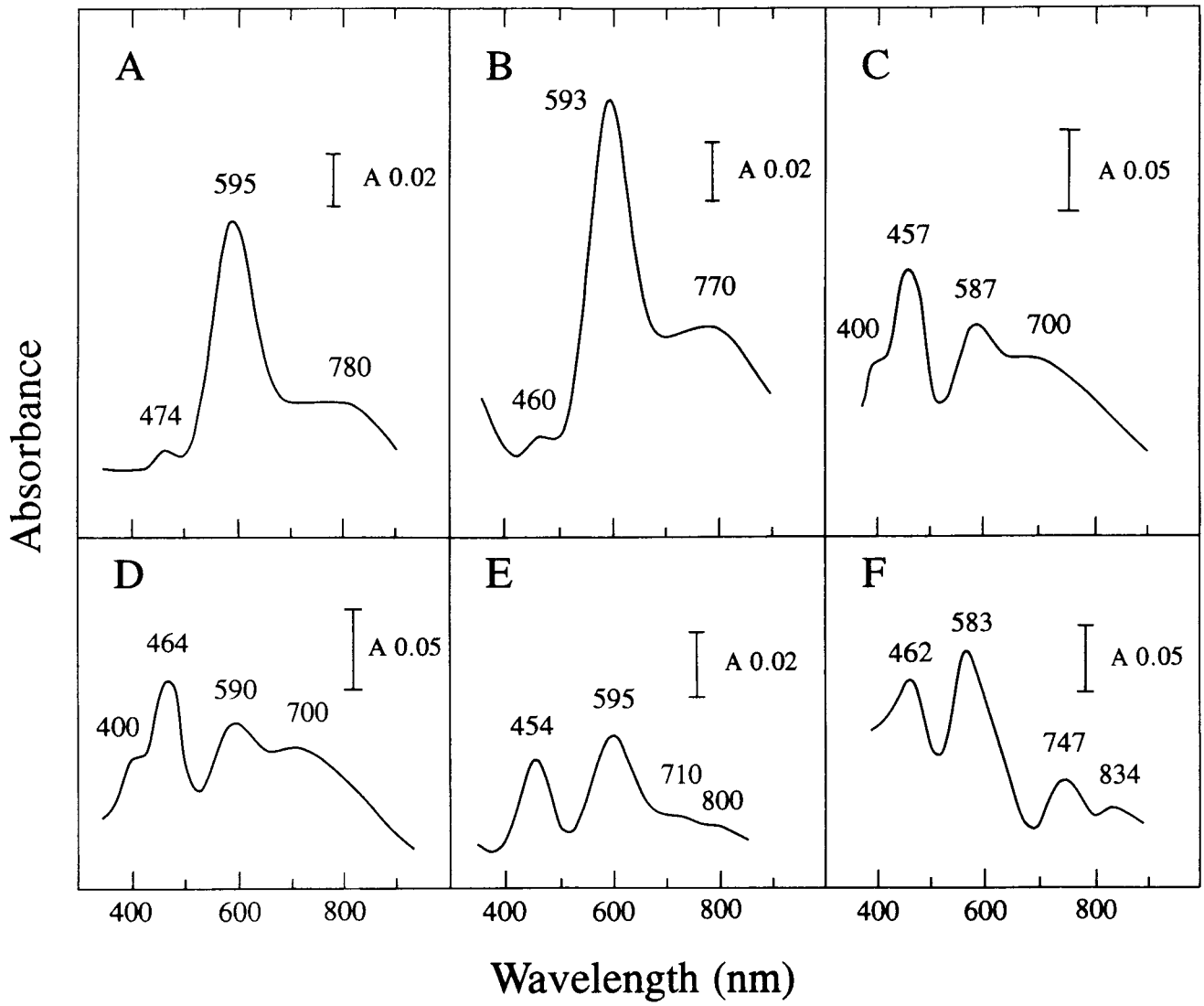

FIG. 11. Electronic absorption spectra of Cu-containing nitrite reductases. (A and B) Blue reductases from *P. aureofaciens* (977) and *Alcaligenes xylosoxidans* (1), respectively. (C through F) Green reductases from *Alcaligenes faecalis* (433) (C), "*A. cycloclastes*" (390) (D), *Bacillus halodenitrificans* (198) (E), and *Rhodobacter sphaeroides* (575) (F). Reproduced with permission from the indicated references.

the electron entry via type 1 Cu, and the active site represented by type 2 Cu.

The formation of a Cu^+—NO^+ nitrosyl complex has been suggested to occur during the reaction of CuNIR with nitrite (371, 803). The cuprous nitrosyl is believed to be the key intermediate in the reaction

$$\text{E-Cu}^+ + \text{NO}_2^- \xrightarrow{+2\text{H}^+,\ -\text{H}_2\text{O}} \text{E-Cu}^+\text{—NO}_2^- \rightleftharpoons \text{E-Cu}^+\text{—NO}^+ \rightleftharpoons \text{E-Cu}^{2+} + \text{NO}$$

The above reaction was formulated initially for the type 1-only nitrite reductase (803), but it is equally valid for the enzyme having its complete set of metal centers (396). The observed reactivity of the enzyme with hydroxylamine favors the existence of an E-Cu^+—NO^+ intermediate (371). When N_2O formation by CuNIR occurs, it is thought to result from the reaction of the nitrosyl intermediate with nitrite or the rebound product NO.

Evidence from ^{1}H ENDOR spectroscopy indicates that nitrite displaces a water molecule from the type 2 Cu site. The lack of nitrogen coupling from $^{14}NO_2^-$ versus $^{15}NO_2^-$ as the substrate can be interpreted that nitrite binds to Cu via its oxygen atoms (366). The spectroscopic data are supported by the structural evidence obtained from nitrite-soaked crystals. Nitrite occupies the site of the water molecule with the oxygen atoms directed slightly asymmetrically toward the type 2 Cu at distances of 2.1 and 2.4 Å (4). The active site is located in a pocket formed by apposition of domains I and II of two different monomers with the lining of domain II by hydrophobic amino acids and that of domain I by more hydrophilic residues. The mechanism is proposed to involve the displacement of a water molecule by nitrite, which leaves the active site as OH^- along the hydrophilic side, where it picks up a proton. The other proton stays on Asp98. This residue is assumed to be

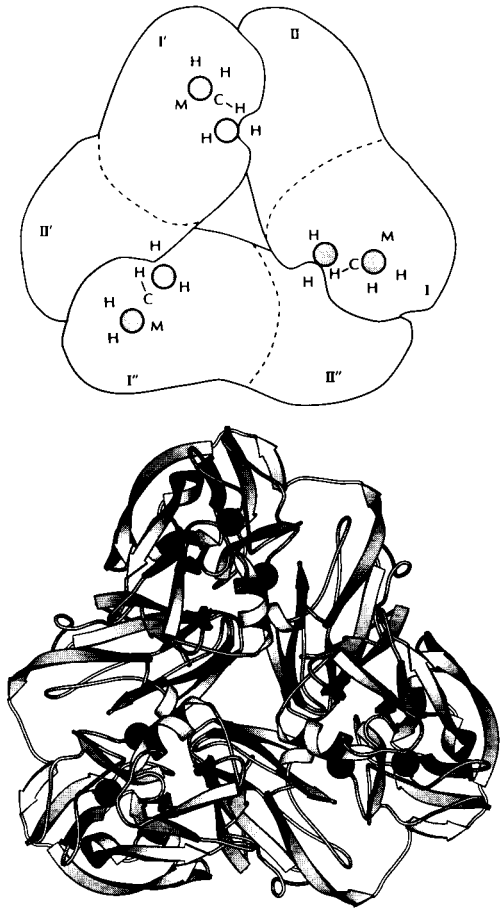

FIG. 12. Cu-containing nitrite reductase from "*A. cycloclastes*." (Top) Schematic representation of the trimer and position of Cu atoms. Full circles represent the six Cu atoms coordinated by cysteine (C), histidine (H), and methionine (M). Domains I and II are related to domains I′, I″ and II′, II″ by a crystallographic threefold axis. The type 2 Cu is bound by three histidines between the subunit interfaces. (Bottom) Polypeptide fold of the CuNIR trimer. Filled circles denote positions of the Cu atoms. Courtesy of E. T. Adman.

essential in protonating an oxygen from nitrite left at the Cu on cleavage of the Cu-ONO adduct. NO exits along the hydrophobic side of the active site pocket (4).

The reactivity of CuNIR with nitrite is mimicked by a synthetic mononuclear Cu^{+}—NO_2^{-} complex that yields NO on protonation by glacial acetic acid (312). Different from the evidence of *O*-NO_2^{-} coordination by the enzyme, the nitrite coordination in the Cu complex is N-bonding, Cu^{+}—*N*-NO_2^{-}. Nitrite could bind initially via its oxygen atoms as observed in the crystal, but rearrange during the catalytic cycle to give the Cu^{+}—NO^{+} intermediate. *O*-NO_2^{-} coordination has also been observed for active-site analogs. Questions remain whether nitrite binds in the enzyme initially to Cu(II) or Cu(I), the identity of the leaving group from the Cu-NO_2^{-} adduct, and whether oxygen transfer to Cu occurs. This point is also relevant to the mechanism of N_2O reduction by the Cu-containing N_2O reductase.

Electron donation. The redox potential for CuNIR from *Alcaligenes xylosoxidans* is +260 mV at pH 7.2 (539). The measured midpoint potential is that of type 1 Cu, since type 2 Cu was absent from the enzyme preparation. For the "*A. cycloclastes*" reductase, with both types of Cu, a midpoint potential of +240 mV was reported for the type 1 Cu (463) and +260 mV was reported for the type 2 Cu (802). The principal electron donors to CuNIR are azurin and pseudoazurin, while cytochromes appear less frequently involved. Azurins and pseudoazurins are distinguished by their optical spectrum and a number of structural elements, but both are members of the cupredoxin family of small (12- to 14-kDa) electron transfer proteins with a single type 1 (blue) Cu atom (2). Pseudoazurins have additional absorption bands around 450 and 750 nm. Azurins have a cysteine at the third position from the N terminus that is part of a disulfide bridge.

Electrons enter CuNIR from a cupredoxin via the type 1 Cu. The intramolecular electron transfer rate from type 1 Cu to type 2 Cu, studied by pulse radiolysis, is slower than the reduction of the type 1 Cu (802). The intramolecular electron transfer path in CuNIR shows analogies to that of ascorbate oxidase and involves the cysteine of type 1 Cu and His135 of the type 2 Cu of the same subunit (488).

Azurins are electron donors for the blue variants of CuNIR found in *P. aureofaciens* (977) and *Alcaligenes xylosoxidans* (209). The latter bacterium synthesizes two similar azurins distinguishable in their primary structure but having the same redox potential (+305 mV) (209). Both proteins are active as electron donors. The spontaneously type 2 Cu-depleted enzyme does not react with azurin but instead is active with reduced cytochrome *c* (583). Cytochrome c_2 is the electron donor to CuNIR of *Rhodobacter sphaeroides* IL106 (720). Pseudoazurins are the electron donors for the green enzymes from *Alcaligenes faecalis* S-6 (432) and "*A. cycloclastes*" (516). The cupredoxin-like donors have similar positive redox potentials as CuNIR to effect the electron transfer nearly isopotentially.

The interaction of the electron donor with its cognate reductase involves a strong electrostatic element. This has been demonstrated by studying the docking of pseudoazurin and CuNIR by site-directed mutagenesis, which revealed a set of appropriately positioned complementary charges on the two proteins (489, 490). Pseudoazurin of *Alcaligenes faecalis* has a surface ring of lysine residues around the Cu atom (Lys10, Lys38, Lys57, and Lys77), which is required for the electron transfer to its cognate CuNIR. There is little change in the rate of electron transfer on substitution of these residues, but the K_m increases substantially, particularly when Lys10 and/or Lys38 is replaced. Lys10 is crucial for electron transfer in this system. At the CuNIR surface, the residues Glu118, Glu197, Glu204, and Asp205 are conserved. Their substitution lowers the enzyme activity and increases the K_m. The following pairing is possible: Glu197 with Lys10, Glu118 with Lys77 and/or Lys57, and Glu204 and/or Asp205 with Lys38. This pairing places the two type 1 Cu sites 14 to 15 Å away from each other. The electron transfer is thought to involve hydrogen bonds along a pathway that shows similarity to that at the Cu_A center of COX (490). In addition to a distinct charge recognition between the molecules, the overall charge distribution of the molecule is also important for the orientation of the molecule toward the reductase (489).

The X-ray structures of pseudoazurin of *Alcaligenes faecalis* and azurin of *P. aeruginosa* are largely superimposable, although there is as little as 11% sequence identity between both proteins (491). In spite of the structural similarity, *P. aeruginosa* azurin reacts poorly with CuNIR from *Alcaligenes faecalis*. This can now be rationalized by the lack of the surface lysines. Introducing two lysine residues increases the affinity of azurin to the *Alcaligenes* reductase (491). It has also been noted that of the relevant charged residues for docking on CuNIR, only Asp205 is conserved in the enzyme of *P. aureofaciens*. The

TABLE 6. Properties of cytochrome cd_1 nitrite reductases (EC 1.9.3.2)

Property	Value for:			
	Pseudomonas aeruginosa	*Pseudomonas stutzeri*[a]	*Paracoccus denitrificans*	*Thiobacillus denitrificans*
Molecular mass (kDa)	119–121[b]	119–134[c]	120	118[b]
Mass (kDa) by SDS-electrophoresis	63	60–65	61.2	65–67
Sequence-derived M_r	60,204	59,532	63,144	
Quaternary structure	α_2	α_2	α_2	α_2
pI	6.9	5.4	3.9	
VIS absorbance (nm) (reduced form)	280, 418, 460, 521, 549, 554, 625–655	278, 417, 460, 522, 548, 554, 625–655	280, 418, 460, 521, 547, 553, 625–655	280, 418, 460, 523, 549, 553, 562, 650
ε_{410}^{ox} (mM^{-1} cm^{-1})	282			312
Purity index^{ox} ($A_{410/280}$)	1.21	1.2	0.75	1.52
EPR parameters (*g* values)				
Heme D_1	2.51, 2.43, 1.71	2.56, 2.42, 1.84		2.50, 2.43, 1.70
Heme C	3.01, 2.29, 1.40	2.97, 2.24, 1.58		3.6
Electron donor(s)	Cytochrome c_{551}, azurin	Cytochrome c_{551}	Cytochrome c_{550}, pseudoazurin	
K_m for NO_2^- (μM)	53		6	
K_m for O_2 (μM)	28		80	
Activity ($\mu mol \cdot min^{-1} \cdot mg^{-1}$)				
With NO_2^-	3.78	4.15	4.0	
With O_2	1.3			
Reference(s)	300, 305, 592, 766, 770, 799, 929		185, 607, 620, 822	375

[a] Unpublished data for strain ZoBell; similar properties are exhibited by cytochrome cd_1 from strain JM300 (896).
[b] By sedimentation velocity or equilibrium.
[c] By gel filtration or nondenaturing gradient electrophoresis.

interaction of an azurin with its cognate CuNIR may therefore be of a different nature (490).

Cytochrome cd_1

Enzyme properties. Cytochrome cd_1 was discovered by Horio et al. during studies on bacterial respiration and was initially described as a cytochrome oxidase from *P. aeruginosa* (362). The synthesis of this oxidase was dependent on nitrate, and Yamanaka (929) established its physiological function as that of a nitrite reductase for the denitrification process. The enzyme, which is structurally simple compared to COX, was long seen as a model for oxygen activation and electron transfer, and the designation "green cytochrome oxidase" has been used frequently in studies about this enzyme in the past. The early work with cytochrome cd_1 has been reviewed by Yamanaka and Okunuki (932), and the ensuing developments have been reviewed by Henry and Bessières (332), Yamanaka (929), and Silvestrini et al. (769). Most of the work carried out on cytochrome cd_1 made use of the enzyme from *P. aeruginosa*, which is one of the best studied denitrification enzymes.

Cytochrome cd_1 has been purified from the sources listed in Table 4, where, except for *Magnetospirillum* (formerly *Aquaspirillum*) *magnetotacticum* and *Roseobacter* (formerly *Erythrobacter*) *denitrificans*, the protein has been assigned the role of the respiratory nitrite reductase from biochemical evidence and in a few cases from corroborating genetic evidence. The enzyme is a homodimer with a subunit mass of around 60 kDa. The properties of cytochrome cd_1 from representative sources are summarized in Table 6. The prosthetic groups are heme C and heme D_1, which are both present in each subunit to render cytochrome cd_1 a tetraheme protein. Cytochrome cd_1 was the first cytochrome for which the presence of two distinct heme groups was established. The spectral properties of cytochrome cd_1 are shown in Fig. 13. The electronic spectrum of the reduced enzyme is quite characteristic due to the long wavelength absorption band of heme D_1, the split α peak, and the weak Soret absorption maximum around 460 nm. The spectroscopic properties of heme D_1 are substantially affected by the two oxo groups and the C-17 acrylate substituent on the macrocycle (Fig. 14).

Cytochrome cd_1 catalyzes the oxidation of cytochrome c_{551} in reducing nitrite to NO (933). The nitrite-reducing activity is inhibited by CN^- but insensitive toward CO, whereas oxygen reduction is sensitive to both inhibitors (769). Mutants of *P. stutzeri* that have lost cytochrome cd_1 can no longer utilize nitrite, and they provided the genetic evidence that respiratory nitrite reduction is intrinsic to this heme protein (972). The oxidation of cytochrome cd_1 by oxygen is about 100-fold slower than that by nitrite (772). The active site for oxygen reduction is heme D_1 (752). Both water and peroxide have been described as the products of oxygen reduction (382, 823). Minor, probably nonphysiological reactivities of cytochrome cd_1 are the slowly proceeding oxidation reduction reactions of CO to CO_2 and of hydroxylamine to ammonia.

Heme D_1, as the noncovalently bound heme, is extractable from nitrite reductase by acidified acetone and can be reintroduced, restoring activity and the spectroscopic properties of the protein. Insertion of synthetic heme D_1 leads to an active enzyme with an electronic spectrum practically identical to that of the native protein, indirectly corroborating the chemical structure of heme D_1 (896). Native heme D_1 can also be replaced by other porphyrins. For instance, the introduction of heme A gives 5% of the oxidase activity of the wild-type enzyme (337).

In the photosynthetic bacterium *R. denitrificans*, cytochrome cd_1 may indeed play the role of a respiratory enzyme, reducing O_2 to water (212). The bacterium aerobically synthesizes two forms of cytochrome cd_1 which are spectroscopically distinguishable and differ by about 1 unit in pI and 100 mV in the redox potentials of heme D_1 (+134 versus +234 mV). They

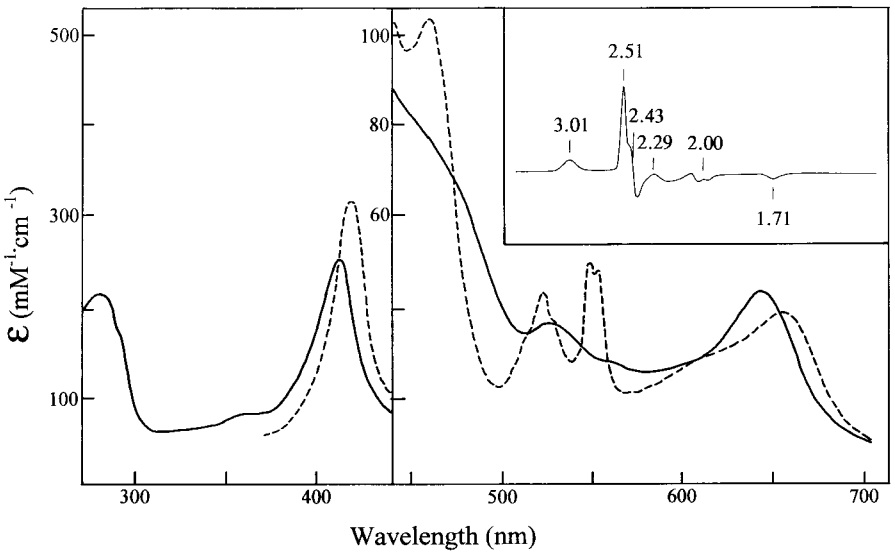

FIG. 13. Electronic absorption and EPR spectra of cytochrome cd_1 nitrite reductase from *P. aeruginosa*. The enzyme was in 0.1 M phosphate buffer (pH 7.0). Solid line, oxidized form; dotted line, ascorbate-reduced form. Reproduced with permission from reference 769. (Insert) X-band EPR spectrum of cytochrome cd_1 of *P. aeruginosa*. The enzyme was in 0.1 M HEPES buffer (pH 8.0). Conditions of recording: microwave power, 2 mW; temperature, 12 K; modulation amplitude, 0.63 mT. Reproduced with permission from reference 799.

also show small differences in their amino acid composition. The oxygen reductase activity of both species is considerably higher than their nitrite reductase activity. Active electron donors are the indigenous cytochromes c_{551} and c_{552}, and mitochondrial cytochrome *c*.

A functionally different type of cytochrome cd_1 has been isolated from *M. magnetotacticum* (934). Magnetotactic bacteria live in soil, freshwater, and salt water; they are motile and morphologically and metabolically diverse. They deposit Fe_3O_4 in intracellular, membrane-enveloped magnetosomes, which allows them to orient in the magnetic field. Cytochrome cd_1 is preferentially synthesized by cells that form magnetite crystals but much less by nonmagnetic cells, which suggests a relationship between denitrification and magnetite mineralization. The environments of hemes C and D_1 of the *Magnetospirillum* enzyme are different from those of the other cytochromes cd_1. Although the long-wavelength absorption is clearly present, the spectral split in the α band of heme C is absent, and the Soret band of heme D_1 is of very low intensity. Modified protein environments are also suggested by the lower redox potentials both for heme C (+191 mV) and for heme D_1 (+180 mV) compared to those in other cd_1-type cytochromes. The enzyme shows nitrite reductase and oxygen reductase activity with artificial electron donors but is inactive with either the indigenous cytochrome c_{550} or the cytochrome c_{551} from *P. aeruginosa*. Like other nitrite reductases, the enzyme is located in the periplasm and is thought to act here as the physiological Fe(II):nitrite oxidoreductase for magnetite formation.

Structure. The primary structure of cytochrome cd_1 has been determined for *P. aeruginosa* both from the *nirS* gene and by protein sequencing (770), and for two strains of *P. stutzeri* (430, 774) and three strains of *Paracoccus denitrificans* (185, 261, 620) from the *nirS* genes. The degree of sequence conservation among the distinct genera is high (≈70%) and groups the NirS proteins as homologous members of a single family (Fig. 3). The *nirS* genes are part of gene clusters that also harbor genes for heme D_1 biosynthesis. The *nir* clusters in turn are linked to genes encoding proteins for NO reduction (Fig. 2).

Cytochrome cd_1 crystallizes readily. The first successful attempt dates back to 1962 (931), and several others have been reported since, yet the protein isolated from strain GB17 of *Paracoccus denitrificans* was the first to yield high-quality crystals for X-ray diffraction (261). Structure determinations of the cytochromes cd_1 from *P. aeruginosa* and *P. stutzeri* are under way. The 1.55-Å crystal structure from *Paracoccus denitrificans* confirmed the dimeric nature of the protein with a heme C and a heme D_1-binding domain in each monomer (Fig. 15). Protein cleavage by subtilisin of the *P. aeruginosa* enzyme had previously shown that the heme D_1 domain (≈48 kDa) can be separated from the heme C domain (364). The isolated heme D_1 peptide retains oxygen reductase activity with ascorbate as the electron donor, but it no longer oxidizes cytochrome c_{551}. Addition of the isolated heme C domain does not restore cytochrome *c* binding, which can be interpreted as being due to a low affinity between the two domains (768). In the *Paracoccus* structure, 20 hydrogen bonds and salt bridges and an axial ligand for heme D_1 make contacts between the two domains (261). Heme D_1 is close to the domain interface (Fig. 15). The shortest heme edge-to-edge distance is 11.0 Å, and the heme C to heme D_1 iron-to-iron distance is 20.6 Å. All intersubunit iron-to-iron distances are at least twice as large.

The *Paracoccus* cytochrome cd_1 structure reveals a predominantly α-helical contribution (12% of the entire molecule) for the heme C domain (amino acid residues 1 to 134); the heme D_1 domain (amino acid residues 135 to 567) consists of a regular β-propeller structure with eight blades (34% β-structure) (261). Viewed end-on, heme D_1 is located at the rotor axis of the propeller-like structure (Fig. 15). Secondary-structure analysis predicts for the *P. aeruginosa* enzyme about a 16% α-helix content concentrated at the N-terminal region and a 48% β-sheet content for the rest of the protein (769). This suggests an overall structure like that of the *Paracoccus denitrificans* GB17 enzyme. Because NirS proteins have a large number of identical positions in their amino acid sequences,

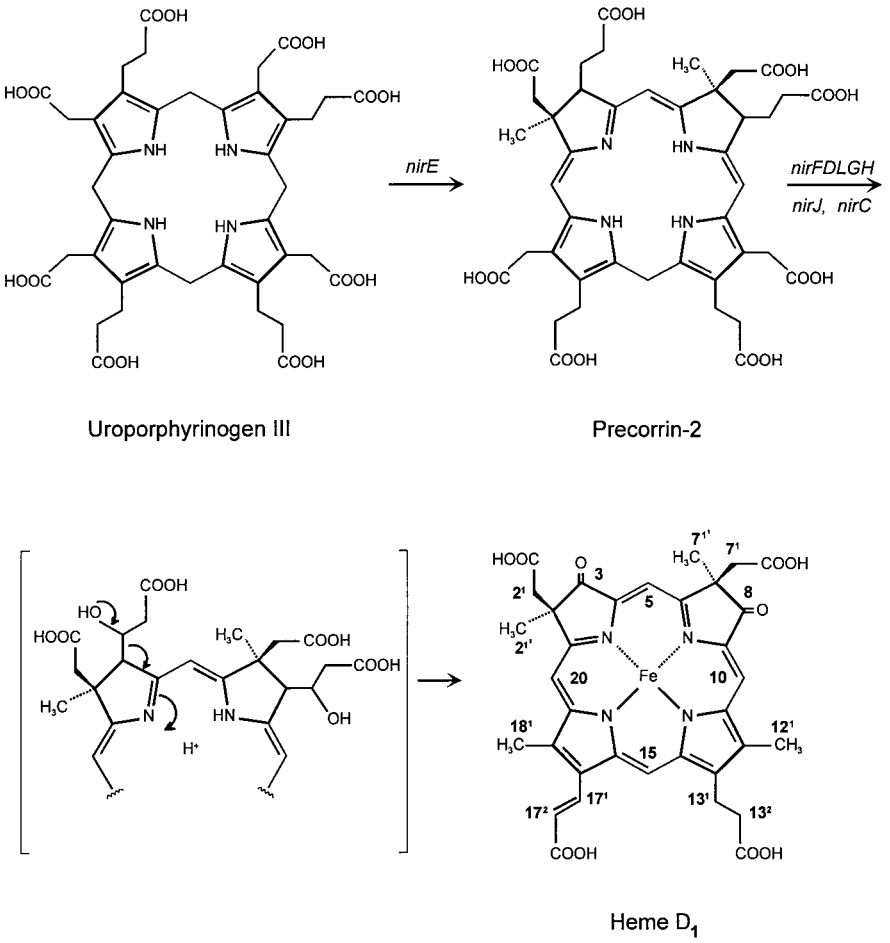

FIG. 14. Chemical structure of heme D_1 of cytochrome cd_1 nitrite reductase and putative heme D_1 precursors. A pathway involving uroporphyrinogen III and precorrin-2 is proposed from genetic evidence (185, 634). See the text for a discussion of the gene products involved. A hypothetical intermediate in the generation of the two oxo groups is shown in brackets. Acid-catalyzed decarboxylation may occur with this intermediate or precorrin-2 (577). The gene products involved in the biosynthetic pathway and their functions require biochemical proof. Numbering of atom positions is according to International Union of Pure and Applied Chemistry/International Union of Biochemistry nomenclature.

similarly folded heme domains are anticipated in the pseudomonadal enzymes. The β-propeller fold is also found in a number of other enzymes, for example methanol dehydrogenase and pyrroloquinoline quinone-dependent alcohol dehydrogenase, not related in primary structures to cytochrome cd_1 (34). The NirF and NirN proteins have recognizable sequence relatedness to NirS and are candidates for similarly folded proteins.

While the heme C domain shows in its predominantly α-helical structure a resemblance to the class I of small *c*-type cytochromes (which carry the heme-binding site N terminally like cytochrome cd_1), the heme coordination is different. The axial ligation of heme C in the crystal structure is *bis*-histidine. Ligation of heme D_1 is by tyrosine (Tyr25) and histidine (His200) (261). The tyrosyl ligand to the heme D_1 domain is provided by a protruding loop from the heme C domain. The corresponding region is well conserved in NirS of two other strains of *Paracoccus* but not in *P. aeruginosa* and is clearly deleted in two strains of *P. stutzeri* (430, 774) and in *R. eutropha* (672). Mutation of Tyr10 to phenylalanine of the *P. aeruginosa* cytochrome cd_1, which is topologically homologous to Tyr25 of *Paracoccus*, does not affect the enzymatic and spectroscopic properties of the enzyme, excluding this residue from the catalytic pathway (170).

The *Pseudomonas* structures are awaited with much interest since they will provide comparative information about heme ligation in cytochrome cd_1. The apparent variation in essential ligands runs counter to the generally held view that residues of active centers are conserved in the primary structure. The oxidized *Paracoccus denitrificans* GB17 structure may represent that of a resting enzyme. Heme ligation of the solution form of this enzyme is unchanged versus the crystal (142). However, reduction of this enzyme changes the His-His ligation of heme C to His-Met and releases Tyr25 from heme D_1 (905). Spectroscopic data obtained with the *P. aeruginosa* enzyme by EPR (592), nuclear magnetic resonance spectroscopy (821), and MCD (889) have previously shown that both heme C and heme D_1 are low spin in the oxidized form and that heme D_1 changes to high spin when reduced. The axial ligands in the oxidized *P. aeruginosa* enzyme are His-Met for heme C and His-His for heme D_1 (799). Recently, it has been pointed out that due to the lack of an appropriate model system, the assignment of MCD bands to specific ligands of heme D_1 is still problematic. With this caveat in mind, a His-Tyr or His-hydroxide coordination is compatible with the EPR and MCD data for the *P. stutzeri* cytochrome cd_1; heme C is clearly ligated by His-Met (142).

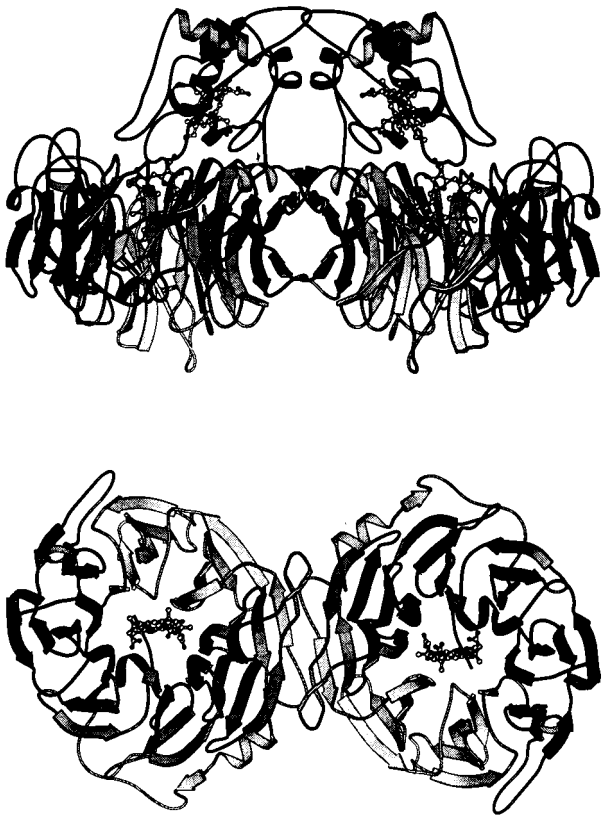

FIG. 15. Polypeptide fold and subunit structure of cytochrome cd_1 nitrite reductase from *Paracoccus denitrificans* GB17 (formerly "*T. pantotropha*"). (Top) Side view of the protein dimer with the heme C domain on top. (Bottom) End-on view of the heme D_1-binding domain with heme D_1 at the axis of an eight-bladed propeller-like structure. Reproduced with permission from reference 261.

Electron donation. Cytochrome cd_1 is an excellent system to study the kinetics of intramolecular electron transfer between the two heme groups and intermolecular transfer in a tripartite system of the enzyme and its electron donors. The redox potential of heme C in the *P. aeruginosa* enzyme is about +290 mV. It is more negative when CO is bound to heme D_1 and more positive when NO is bound (reviewed in reference 769). The heme C potential increases by more than 100 mV in the heterologously expressed protein that lacks heme D_1; together with the data on selectively bound ligands, this clearly shows that there is an interaction between both heme centers affecting the redox potential (767). The redox potential of heme D_1 (+287 mV) is close to that of heme C (726), but values above and below that of heme C have also been found, suggesting that a more rigorous treatment of this aspect is required (769).

Cytochrome cd_1 has a dual electron acceptor specificity for cytochrome c_{551} and azurin in vitro (638, 900). The two electron donors are interchangeable and exist in rapid redox equilibrium with each other (765). Cytochrome c_{551} is a more efficient electron donor in vitro than azurin is (771). High-resolution X-ray structures and solution structures obtained by nuclear magnetic resonance spectroscopy have been determined for cytochrome c_2 (c_{550}) of *Paracoccus denitrificans* (63) and *Rhodobacter sphaeroides* (32), and cytochrome c_{551} of *P. aeruginosa* (202, 549) and *P. stutzeri* (112, 113). A mutation in *cycA* (cytochrome c_{550}) of *Bradyrhizobium japonicum* generates a phenotype for anaerobic growth on nitrate, pointing at a distinct electron acceptor protein somewhere in the denitrification pathway of this bacterium; mutations in *cycB* (encoding cytochrome c_{552}) and/or in *cycC* (encoding cytochrome c_{555}) leave nitrate-dependent growth unaffected (96).

Cytochrome cd_1 of *P. aeruginosa* has been subjected to extensive screening for its reactivity with bacterial and eukaryotic *c*-type cytochromes. It reacts only sluggishly with eukaryotic cytochromes *c*, with the exception of the teleost proteins, which give full oxidase activity. For other heterologous cytochromes, the nitrite reductase activity is best with algal cytochrome c_6 (929). The interspecies cross-reactivity of cytochromes has also been studied with cytochrome cd_1 from *Paracoccus denitrificans* and *Alcaligenes faecalis* (822). The heterologous cross of *Alcaligenes* cytochrome c_{554} with *P. aeruginosa* cytochrome cd_1 is the only one where electron transfer to nitrite is more rapid than in the homologous combination. Cytochrome c_{551} from *P. aeruginosa* is inactive with *Alcaligenes* cytochrome cd_1 (822).

In spite of the ample in vitro evidence for azurin as an electron donor to cytochrome cd_1, recent findings cast doubts on a principal role of azurin in the denitrification process of *P. aeruginosa*. A mutation in the *azu* gene is silent with respect to nitrite reduction; the cellular growth rate is affected only in an azurin-cytochrome c_{551} double mutant (880). The expression pattern of azurin is not well correlated with the expression of denitrification as it is synthesized constitutively under aerobic conditions, in part also anaerobically under the control of ANR, and is maximally expressed in the stationary phase independent of nitrate or nitrite.

To establish the physiological roles of cytochrome c_{551} and azurin on the basis of genetic evidence is complicated because of the availability of more than one electron donor and their interchangeability. A mutation in cytochrome c_{550} of *Paracoccus denitrificans* is silent with respect to nitrite reduction (872); cytochrome cd_1 from *P. denitrificans* GB17 accepts electrons both from its indigenous pseudoazurin and from cytochrome c_{550} (585). The above findings demonstrate a certain ambivalence in the recognition of electron donors for nitrite reductase, which involves both specific and generic elements. To better describe this phenomenon, the term "pseudospecificity" has been suggested (906).

A hydrophobic patch is important for the electron transfer from azurin to cytochrome cd_1 (861). The Cu ligand His117 is located in the center of this patch and protrudes from the protein surface, making it the likely site of electron entry and exit (868). Azurin has been the subject of extensive analysis by introducing recombinant modifications into this protein. These studies, including the determination of several crystal structures of protein modifications, have provided a most detailed picture of critical amino acid positions affecting electron transfer, midpoint potential, and the type of Cu coordination (118).

The hydrophobic-patch concept for recognition among electron transfer partners has been extended to cytochrome cd_1, pseudoazurin, and *c*-type cytochromes (906). The hydrophobic patch on pseudoazurin comprises 11 amino acids and is located in the middle of the positively charged hemisphere of the protein, whereas the other hemisphere is negatively charged and imprints an overall bipolar charge distribution. The patch is surrounded by 12 basic residues, 11 of which are conserved in other pseudoazurins. The importance of these residues has been discussed above for the interaction of pseudoazurin with a cognate CuNIR. A complementary hydrophobic patch surrounded by negative charges is present on top of the heme C domain of cytochrome cd_1 and can form the docking site for a pseudoazurin molecule. This brings the metal centers to within 19 to 20 Å, and no other arrangement makes them approach more closely (906). Charge neutralization on docking is thought to enhance electron transfer. Hy-

drophobic patches are found also on *c*-type cytochromes and may be important for the docking of cytochromes c_{551} and c_{550} to nitrite reductases. Not being highly discriminatory, this type of recognition provides a rationale for the interchangeability of electron donors observed among denitrification components.

Mechanistic aspects. A stoichiometric reaction mechanism involving the sequential activity of cytochrome cd_1 and NO reductase is now supported by most of the biochemical and genetic evidence. Isolated cytochrome cd_1 was never shown to produce N_2O as the major product. The rate of N_2O formation is also much lower than that of NO formation. The mechanism by which some N_2O is formed by nitrite reductase may involve dimerization of a nitroxyl (269). Alternatively, isotope fractionation and ^{18}O exchange data (748) support a mechanism of two nitrite molecules reacting in sequence with the enzyme and an activated intermediate (895). The physiological reaction of cytochrome cd_1 is the protonation of nitrite and removal of water to yield NO:

$$NO_2^- + 2H^+ + e^- \rightarrow NO + H_2O$$

$$[E_0'(\text{pH } 7.0) = +0.37 \text{ V}; \Delta G^{\circ\prime} = -76.2 \text{ kJ/mol}]$$

Cytochrome cd_1 causes the nitrosation of hydroxylamine, azide, and amines (268, 452). The nitrosation of secondary amines may be related to the carcinogenic potential of nitrite (115). Further, cytochrome cd_1 catalyzes ^{18}O exchange between nitrite and water (268, 943). All these above activities are intimate parts of the catalytic mechanism, which is proposed to involve the strong nitrosyl donor d_1-Fe^{2+}—NO^+ (268, 452). Fourier transform infrared spectroscopy of cytochrome cd_1 of *P. stutzeri* JM300 exhibits absorption bands at 1,910 and 1,874 cm^{-1} that have been ascribed to this species (891). Reduction of Fe^{2+}—NO^+ yields the heme D_1 nitrosyl complex, Fe^{2+}—NO, observed on direct incubation of the reduced protein with NO (591). Under physiological conditions, NO is thought to leave from an Fe^{3+}—NO species in equilibrium with the Fe^{2+}—NO^+ intermediate (31, 891):

$$d_1\text{-}Fe^{2+} + NO_2^- \rightarrow d_1\text{-}Fe^{2+}\text{—}NO_2^- \overset{+2H^+,\ -H_2O}{\rightleftharpoons}$$

$$d_1\text{-}Fe^{2+}\text{—}NO^+ \leftrightarrow d_1\text{-}Fe^{3+}\text{—}NO \rightleftharpoons d_1\text{-}Fe^{3+} + NO^{\bullet}$$

The reaction kinetics of the enzyme with nitrite have been studied by stopped-flow and EPR spectroscopy (772). The initial reaction of the reduced enzyme with nitrite is very fast and within the 4-ms dead time of the experimental setup. The estimated minimal second-order rate constant $k \geq 10^8\ M^{-1}\ s^{-1}$ is much greater than the rate constant for the reaction of the enzyme with oxygen, $3.3 \times 10^4\ M^{-1}\ s^{-1}$, reinforcing its role as a nitrite reductase (772). Perhaps with the exception of the *Roseobacter* enzyme, there is no evidence that oxygen reduction by cytochrome cd_1 is of physiological relevance in a denitrifying bacterium. The ascorbate-reduced cytochrome cd_1 has one low-spin and one high-spin heme per subunit; the latter is assigned to a pentacoordinated heme D_1. Reduction of cytochrome cd_1 thus provides a free coordination site for nitrite binding and its subsequent reduction. The crystal structure shows that both the substrate, nitrite, and the reaction product, NO, bind via their nitrogen atoms to the iron of heme D_1, which is the catalytic site of cytochrome cd_1 (905). Protonation and dehydration of nitrite are also fast and still unobservable within the experimental dead time. The first spectroscopically detectable species is NO bound to oxidized heme D_1. The final species is the fully reduced enzyme, with NO remaining bound to heme D_1 under the conditions studied (pH 8.0) (772). To bring about the discharge of NO from the enzyme, a conformational flexibility of cytochrome cd_1 during redox cycling may be required. For *Paracoccus denitrificans* GB17 cytochrome cd_1, the axial tyrosine ligand Tyr25 of heme D_1 was shown to move on redox cycling and is postulated to displace bound NO from heme D_1 (261, 905). The adjacent histidines His345 and His385 are thought to facilitate the protonation of heme D_1-coordinated nitrite.

ANAEROBIC HEME AND HEME D_1 BIOSYNTHESIS

Heme D_1 of cytochrome cd_1 consists of an unusual macrocycle with a set of oxo, methyl, and acrylate substituents, making it distinct for denitrification and unique among the tetrapyrroles (Fig. 14). Initially, heme D_1 had been proposed to have the structure of an acrylochlorin. On reassessment of the structural data, the high similarity to those of isobacteriochlorines led to the formulation of heme D_1 as a dioxo-isobacteriochlorine or, more precisely, a 3,8-dioxo-17-acrylate-porphyrindione (135, 136). The structure of the macrocycle has been proven from chemical synthesis (577, 921). Rings A and B deviate slightly from planarity in the crystal structure of heme D_1; the plane of the acrylate side chain is tilted by 24° with respect to ring D to minimize steric repulsion between H-17 and C-18^1-CH_3 (589). Isobacteriochlorines are easier to oxidize than diones and other porphyrins, underlining the fact that porphyrindiones are not just isobacteriochlorines with additional oxo groups (13). The distinct redox properties of heme D_1 are assumed to be due to the oxo groups in conjugation with the macrocycle. Atomic distances derived from a 2,7-dioxo-octaethylpophyrin model for heme D_1 suggest a dome-like shape with a pentacoordinated high-spin Fe(III) outside of the ring plane (38).

Anaerobic Control of Heme Biosynthesis

Heme biosynthesis starts with the synthesis of ALA. In the alpha subclass of the *Proteobacteria*, it proceeds via the glycine-succinyl-coenzyme A (C-4) pathway, but other groups of bacteria and the archaea use the more prevalent glutamate (C-5) pathway (Fig. 16). The biosynthesis of protoheme has been reviewed previously (175). Within the prokaryotes, much of the relevant work has been done with enteric baceteria. To conform with the purpose of this review, the enzymes and genes for heme biosynthesis from denitrifying bacteria are being emphasized. Relevant findings are also mentioned when the strain under study is not proven to denitrify, since there is no reason to assume that the pathway in the related denitrifying bacterium will be different.

Bacteria that use the C-4 pathway, such as *Bradyrhizobium japonicum*, *Sinorhizobium meliloti*, or *Rhodobacter sphaeroides*, synthesize ALA by the pyridoxal-5′-phosphate-containing ALA synthase (425). The corresponding *hemA* gene from *S. meliloti* was the first cloned gene for bacterial heme biosynthesis (511). It is by unfortunate coincidence that the same gene designation exists for an entirely different gene within the C-5 pathway. Identification of *hemA* genes encoding ALA synthases includes *B. japonicum* (552), *Rhodobacter capsulatus* (363), *R. sphaeroides* (510, 606), and *Paracoccus denitrificans* (629). Under denitrifying conditions, the activity of this enzyme is increased to satisfy the greater demand for porphyrins (485, 804). The rate of ALA synthesis depends on the external factors oxygen and nitrate (373).

The central metabolite of the C-5 path is glutamyl-$tRNA^{Glu}$ formed by glutamyl-tRNA synthetase (EC 6.1.1.17), the same

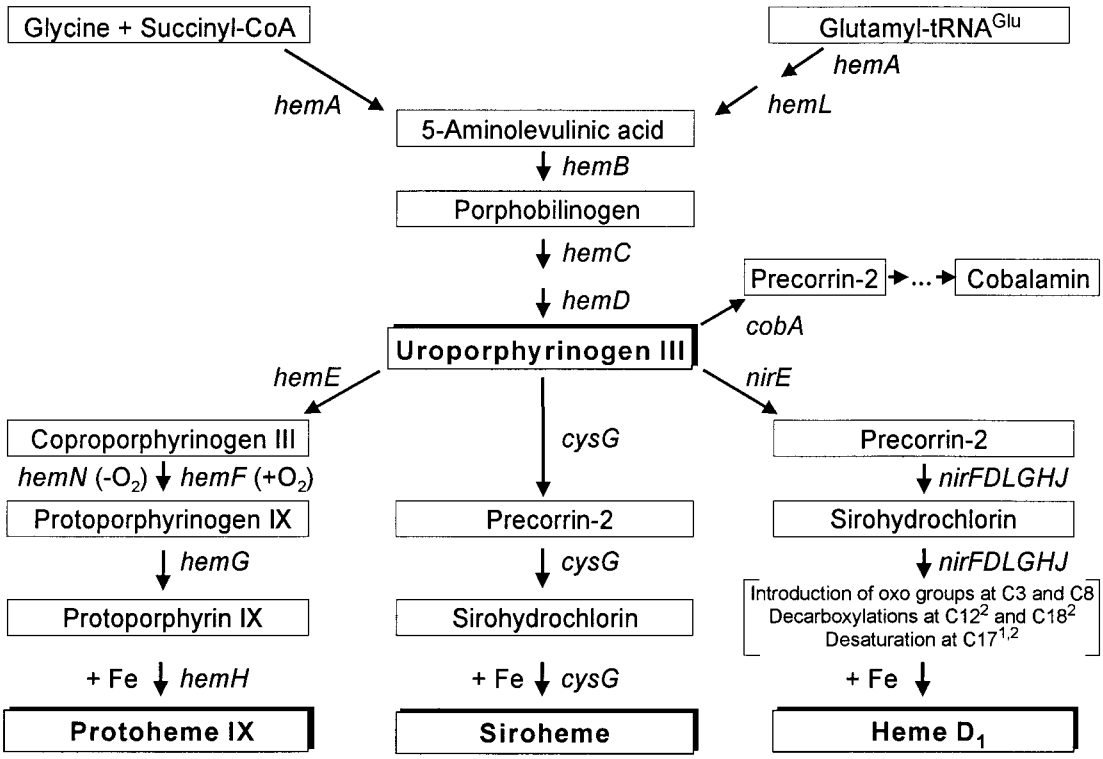

FIG. 16. Pathways of heme D_1 and protoheme IX biosynthesis for cytochrome cd_1. The bacterial gene designations given next to the arrows correspond to the following enzymes: *hemA* (C-4 pathway), ALA synthase (EC 2.3.1.37); *hemA* (C-5 pathway), glutamyl-tRNA reductase (no EC number); *hemL*, glutamate-1-semialdehyde-2,1-aminomutase (EC 5.4.3.8); *hemB*, porphobilinogen synthase (EC 4.2.1.24); *hemC*, porphobilinogen deaminase (EC 4.3.1.8); *hemD*, uroporphyrinogen III synthase (EC 4.2.1.75); *hemE*, uroporphyrinogen III decarboxylase (EC 4.1.1.37); *hemN* (oxygen-independent) and *hemF* (oxygen-dependent) coproporphyrinogen III oxidases (EC 1.3.3.3); *hemG*, protoporphyrinogen IX oxidase (EC 1.3.3.4); *hemH*, ferrochelatase (EC 4.99.1.1); *cysG*, siroheme synthase (EC 2.1.1.107); *cobA* and *nirE*, *S*-adenosyl-L-methionine-uroporphyrinogen III methyltransferases (EC 2.1.1.107). The heme D_1 pathway is hypothetical in the sequence of reaction events (see the text for a discussion). Possible functions of the *nirFDLGH* gene products are listed in brackets. The branch leading from uroporphyrinogen III to (adenosyl)cobalamin involves 15 steps and close to 20 gene products in "*P. denitrificans*" (704). The branch from precorrin-2 to coenzyme F-430 is not included in this scheme.

enzyme as in protein synthesis (Fig. 16). ALA is formed by the reduction of glutamyl-tRNA to glutamate-1-semialdehyde under release of $tRNA^{Glu}$ by glutamyl-tRNA reductase (the *hemA* product), and subsequent transamination of the semialdehyde by glutamate-1-semialdehyde-2,1-aminomutase (the *hemL* product) (399). *P. aeruginosa* and *P. stutzeri* use the C-5 pathway (373, 550). The glutamyl-tRNA synthetase has been studied in the denitrifier *Bacillus stearothermophilus* (102).

Under denitrifying conditions, heme proteins are more abundant in the cell, which requires a steady supply of porphyrins along anaerobic pathways of synthesis. The cytochrome composition changes dramatically when growth conditions are anaerobic or O_2-limited and nitrate is provided to the denitrifiers *P. stutzeri* (462, 517), *R. sphaeroides* IL106 (573), *Paracoccus denitrificans* (95), *A. brasilense* (500), *M. magnetotacticum* (617), or *B. japonicum* (178). One switch point for anaerobic heme synthesis is at the level of ALA synthesis (Fig. 16). The *hemA* transcript (glutamyl-tRNA reductase) of *P. aeruginosa* is increased in denitrifying but not in arginine-fermenting cells. This suggests that nitrate is an inducer and possibly the involvement of a homolog of the nitrate response regulator NarL. The oxygen response is proposed to be mediated via the redox active transcription factor ANR (373). No response of *hemA* toward iron was found. The transcription of *hemL* (glutamate-1-semialdeyde-2,1-aminomutase) also responds to denitrifying conditions (low oxygen and presence of nitrate), although to a lesser degree than that of *hemA* does. It is unaffected in an *anr* mutant, opening the possibility of an alternative redox regulator (372).

Anaerobic control of ALA synthase in *B. japonicum* is exerted through FixLJ and the FNR homolog FixK (627), i.e., the same signal transduction pathway that regulates nitrate respiration in this bacterium. Deletion of the FNR-binding motif in the promoter abolishes *hemA* expression under low-oxygen conditions. At the same time, the *hemA* transcript responds to iron availability, which was the first example of an iron-regulated gene of bacterial heme biosynthesis (626). However, binding of the *E. coli* Fur repressor to the putative *hemA* Fur box could not be demonstrated and leaves the mechanism of the iron response unclear.

ALA is converted to porphobilinogen by the *hemB* product porphobilinogen synthase (ALA dehydratase). The enzyme has been purified from many sources including *Rhodobacter sphaeroides* (310); the *hemB* gene has been isolated from *B. japonicum* (140).

The transformation of porphobilinogen into uroporphyrinogen III is catalyzed by porphobilinogen deaminase (the *hemC* product) and uroporphyrinogen III synthase (the *hemD* product). Uroporphyrinogen III is the central intermediate from which several macrocycles branch into individual pathways (Fig. 16). *P. aeruginosa* is among the bacteria for which *hemC* and *hemD* have been identified (584). Mutants affected in these genes show a nitrate effect with respect to alginate synthesis and suggest a hitherto unknown link between a heme-

dependent process or the energetic status of the cell and mucoidy.

A second anaerobic switch point in heme biosynthesis is the decarboxylation of coproporphyrinogen III to protoporphyrinogen IX with an O_2-independent dehydrogenase (the *hemN* product) instead of the O_2-dependent oxidase (the *hemF* product) (Fig. 16). The gene for the O_2-independent enzyme is relevant for denitrification. It was first isolated from *Rhodobacter sphaeroides* (there also termed *hemF*) (155) and has been studied in more detail with the enteric bacteria (836, 925). Recently, operons with *hemN* homologs with identical genetic organizations were described for *Rhodobacter sphaeroides* and *Paracoccus denitrificans* (871, 955). The *hemN* homologs are transcribed divergently from the *fnr*-like genes *fnrL* and *fnrP* and the downstream contiguous *cco* genes for terminal oxidases of the cbb_3 type. The FNR-like factors are proposed to regulate both the *hemN* and *cco* genes under anaerobic conditions.

The six-electron conversion of protoporphyrinogen IX to protoporphyrin IX by an oxidase (the *hemG* product) requires molecular oxygen in eukaryotes, but in bacteria the enzyme is membrane bound and channels the electrons to the respiratory chain (176, 397). Nitrate and nitrite can substitute for oxygen as electron acceptors (398, 457). An anaerobic pathway to protoheme involving precorrin-2 has been suggested, but the evidence for such a novel route is still inconclusive (7, 86). Key elements in heme biosynthesis therefore also proceed under anaerobic conditions, as required for a denitrifying bacterium. Control is exerted in part by nitrate and low oxygen tension, i.e., central inducers in the denitrification regulatory circuit (see the section on regulation, below).

Biosynthesis of Heme D_1

The distinct steps in the biosynthesis of heme D_1 are beginning to be resolved in terms of reactions, necessary enzymes, and underlying genes. Branching from uroporphyrinogen III, it is most likely that the heme D_1 pathway has precorrin-2 (dihydrosirohydrochlorin) and sirohydrochlorin as intermediates (Fig. 14 and 16). Except for the methyl groups $2^{1'}$ and $7^{1'}$, the entire carbon skeleton of heme D_1 is derived from ALA, favoring a biosynthetic scheme of incorporating four equivalent porphobilinogen molecules into heme D_1. This discards the hypothesis that two porphobilinogen molecules condense with two pyrrole derivatives that provide in their distinct modifications the structures observed in heme D_1 (Fig. 14) (550).

The *nirE* gene, which is part of the *nir* gene clusters (Fig. 2), encodes an *S*-adenosyl-L-methionine-dependent uroporphyrinogen III methyltransferase (EC 2.1.1.107). Mutagenesis of *nirE* results in a semiapocytochrome cd_1 that lacks heme D_1 (185). It is assumed that NirE catalyzes the methylation of uroporphyrinogen III during heme D_1 synthesis, yielding precorrin-2. Dehydrogenation of this intermediate gives sirohydrochlorin (Fig. 16).

In the siroheme branch, the entire pathway from uroporphyrinogen III to siroheme is catalyzed in *E. coli* and *Salmonella typhimurium* by a multifunctional enzyme encoded by the *cysG* locus (894). The NirE proteins of *Paracoccus denitrificans* (185), *P. stutzeri* (283), and *P. aeruginosa* (444) are homologous to CysG but lack its N-terminal domain that catalyzes dehydrogenation and Fe chelation.

The challenge provided by heme D_1 biosynthesis lies in the clarification of how the oxo groups in positions C-3 and C-8 are generated with elimination of the propionate side chains (Fig. 14). Since the reaction proceeds under anaerobic conditions, participation of an oxygenase is unlikely. Diols and epoxides have been suggested as precursors of the oxo groups. Chang considered the origin of the carbonyls from an acid-catalyzed pinacol-pinacolone rearrangement (598) of vicinal hydroxy groups or an epoxide under shift of methyl groups to C-3 and C-8 positions (135). The chemical synthesis of heme D_1 by using protoporphyrinogen IX was based on such a mechanism (921). The methyl groups on C-2 and C-7 have the absolute configuration 2*R*,7*R* as in siroheme, coenzyme B_{12}, and factor F-430 of the methanogens (577). In heme D_1, they are probably derived from methionine as the result of *S*-adenosyl-L-methionine-dependent methylation (937). This finding does not support a mechanism involving a pinacole-type rearrangement but, rather, suggests an unprecedented route for introduction of the oxo groups. Micklefield et al. have suggested a hydroxylation of the propionate side chain followed by a reverse aldol reaction (577) (Fig. 16).

The conversion of precorrin-2 to heme D_1 is likely to be catalyzed by the products of the *nirD* locus (*nirCFDLGH*) (Fig. 2), which has the coding potential for a high-M_r multisubunit complex. A mutation in each single gene of this locus results in the absence of heme D_1 from cell extracts or the enzyme or in a dysfunctional cytochrome cd_1 (185, 443, 634, 938, 972). The function of NirF and its cellular location are unknown. NirF shows weak similarity to NirS, which may hint at a scaffold function for heme D_1 synthesis, provided that it is located in the cytoplasm. The relationship among several NirF proteins is shown in Fig. 3. Redundancy in the duplicated *nirDLGH* genes is not believed to exist, since each gene is essential, and the structurally similar proteins may catalyze repetitive functions in heme D_1 synthesis. Further biochemical and genetic studies are necessary to disclose the precise reactions catalyzed by the gene products of the *nirD* locus.

A further modification required to transform sirohydrochlorin to heme D_1 is the decarboxylation of the C-12 and C-18 acetate side chains on pyrrole rings C and D to yield the methyl groups. Uroporphyrinogen decarboxylase (EC 4.1.1.37) of the protoheme pathway provides a precedent for such a modification (423). The C-17 propionate requires desaturation to give the acrylate substituent. Aside from heme D_1, an acrylate side chain is found on pyrrole ring D of chlorophyll *c* (48). The corresponding enzymes that act on the cognate heme D_1 precursors need to be identified.

The last biosynthetic reaction for a macrocycle is usually the insertion of iron by a chelatase, and this is also assumed to be the last step for heme D_1 synthesis. Ferrochelatase (the *hemH* product) in the protoheme pathway is a membrane-bound, high-molecular-weight enzyme ($M_r \approx 115\,000$) in *Rhodobacter sphaeroides* (174). The subunit mass is much smaller for the *Bradyrhizobium japonicum* enzyme (38.3 kDa) (259), and a soluble variant exists in *B. subtilis* at about 600 molecules per cell (317). Which protein performs the chelatase function in the heme D_1 pathway is unknown.

Beyond the mere synthesis of the macrocycle, there is mutational evidence for functions necessary for the assembly or maturation of cytochrome cd_1. In each case, *nirF* is preceded in the *nir* clusters by *nirC* (Fig. 2), which encodes a putatively periplasmic monoheme cytochrome *c* and whose mutagenesis results in a defective cytochrome cd_1 (185, 938). The *nirJ* and *nirN* genes of *P. aeruginosa*, to which *orf393* and *orf507* of *P. stutzeri* are homologous (283), result on mutagenesis in a Nir^- phenotype without preventing NirS synthesis (444). NirJ and the *orf393* product have similarity to PqqE/PqqIII/Pqq proteins of unknown functions in the biosynthetic pathway of pyrroloquinoline quinone, to NifB of nitrogenase Mo cofactor biosynthesis, and to MoaA of MPT biosynthesis. These proteins have an N-terminal conserved cysteine motif, CxxxCxYC

(835), which is CxxxCxxCY in NirJ. The C-terminal domain of NirJ also is rich in cysteines, as are the MoaA and Pqq proteins, for which a metal-binding site has been postulated.

The NirN protein (*orf507* product) has overall similarity to NirF and NirS (Fig. 3) and is related in its heme C-binding domain to NirC (283, 444). Several of the *nir* gene products presumed to be of biosynthetic or auxiliary function have potential export signals to indicate that the periplasmic compartment participates in the final maturation steps of NirS. Unfortunately, a periplasmic location has not been established experimentally for any of these proteins, precluding a more definite discussion.

The pathways for siroheme, cobalamin, and heme D_1 synthesis share precorrin-2 as an intermediate after uroporphyrinogen III; the siroheme and heme D_1 pathways are likely to have sirohydrochlorin as a second common intermediate (Fig. 16). Coenzyme B_{12} prototrophic denitrifiers that utilize nitrate as the nitrogen source require siroheme as a prosthetic group for their assimilatory nitrite reductase and use all three pathways that involve precorrin-2. The putative methyltransferase, NirE, for the heme D_1 pathway has ≈40% sequence identity to the *cobA* product in the coenzyme B_{12} pathway of "*Pseudomonas denitrificans*" (166). Homologs of *cobA* exist in *P. fluorescens*, *P. aeruginosa* (194), and *B. stearothermophilus* (149). NirE is also similar to CysG of the siroheme pathway. In spite of the existing structural similarities among the methyltransferases, these enzymes discriminate their cognate pathway by responding to different regulatory signals. Heme D_1 synthesis is anticipated to be regulated anaerobically. Both, *nirE* and the putative *nirMCFDLGH* (*P. stutzeri*) and *nirSMCFDLGHJEN* (*P. aeruginosa*) operons have recognition motifs for the anaerobic regulator FNR in their promoter regions. In contrast, the siroheme pathway is nitrogen regulated and independent of oxygen. A mutation in *nirE* results in a recognizable Nir^- phenotype (185), indicating that other methyltransferases cannot substitute for its role. The branch point for the distinct macrocycles thus is assumed to be at uroporphyrinogen III and not at precorrin-2.

Cytochrome *c* Biogenesis

The biogenesis of cytochrome cd_1, like that of the other periplasmic *c*-type cytochromes, involves the translocation of the protein across the membrane, concomitant or separate transport of the prosthetic groups into the periplasm, covalent binding of heme C and insertion of the noncovalent heme D_1, and folding of the protein into its mature form. The order of these events is not rigorously established.

A functional cytochrome cd_1 carrying both types of heme groups cannot be heterologously expressed from *nirS* alone. Expression of *nirS* in *P. putida* (767), *E. coli* or *P. aureofaciens* (282) yields in each instance semiapocytochrome cd_1 with only heme C attached to the protein. The functions required for heme D_1 synthesis apparently are not provided by *E. coli* or the two other hosts tested. However, as judged from NO formation by a cell extract, expression of *nirS* together with ca. 10 kb of the downstream region from *Paracoccus denitrificans* IFO 12442 leads to a functional cytochrome cd_1 in *E. coli* (620). The genes of strain IFO 12442 contributing to heme D_1 biosynthesis have yet to be identified, but it appears from this observation that the entire cytochrome cd_1 biogenesis can be studied in *E. coli.*

In using *E. coli* as the host background for cytochrome cd_1 expression, one is confronted with its innate, aerobically limited capability to synthesize a *c*-type cytochrome. Under anaerobic conditions of nitrate, nitrite, or trimethylamine *N*-oxide respiration, this capacity increases to five different *c*-type cytochromes, with the formate-dependent, ammonifying multiheme nitrite reductase (the *nrfA* product) being one of them (383). Certain genes for a foreign cytochrome *c* are expressed by *E. coli* but, for no obvious reasons, without heme C. Occasionally this deficiency is relieved by expression under anaerobic conditions, as shown for cytochrome c_2 from *Rhodobacter sphaeroides* (555) and cytochrome c_{550} (the *cycA* product) from *Bradyrhizobium japonicum* (819), but it does not provide a general solution to the problem. The overall conformation of the apoprotein was suggested to be among the factors that determine whether a holocytochrome *c* is being synthesized (651).

Cytochrome cd_1 from *P. stutzeri*, which is obtained as semiapocytochrome cd_1 in *E. coli* (282), does meet the requirements necessary for heme C attachment. The translocation of cytochrome cd_1 to the periplasm proceeds in the absence of heme D_1, as can be inferred from the synthesis of a semiapocytochrome cd_1 (972), as well as in the absence of both heme D_1 and heme C (628). The conclusion is that heme attachment occurs at the periplasmic side of the membrane. Indeed, periplasmic heme insertion has been generally suggested for the bacterial *c*-type cytochromes (51). For cytochrome c_{550}, experimental evidence shows that heme attachment follows protein transport to the periplasm (708, 819). This may include signal-peptide cleavage prior to heme ligation. Deletion of the entire signal peptide from cytochrome c_2 still results in the synthesis of a holoprotein and argues for an anchoring to the membrane not being required for heme insertion (98).

The pioneering work with *Rhodobacter capsulatus* and *Bradyrhizobium japonicum* has established that the biogenesis of *c*-type cytochromes requires a considerable number of genes encoding a multifunctional heme transport and heme lyase complex (reviewed in reference 816). Mutations in these genes abolish cytochrome *c* synthesis and consequently are highly pleiotropic (51, 668). The biogenesis of *c*-type cytochromes is a fundamental problem of general biological significance. Since the denitrification process requires the de novo synthesis of a substantial number of heme proteins under anaerobic conditions, it is strictly dependent on this system. The loss of anaerobic growth on nitrate has been reported for cytochrome *c*-deficient mutants of *Bradyrhizobium japonicum* (668), *S. meliloti* (446), and *P. aeruginosa* (669).

Many of the relevant biogenesis genes have been identified during studies of unrelated research topics with different bacteria, for instance nitrogen fixation or Cu homeostasis. Figure 17 shows the organization of homologous gene clusters for cytochrome *c* biogenesis from denitrifiers and nitrate respirers. The operons *cycVWZX* (*Bradyrhizobium japonicum*) (668) and *helABCD* (*Rhodobacter capsulatus*) (51) are presumed to encode ABC transporters for heme, whereas the *cycHJKL* (*B. japonicum*) and *cycH*, *ccl1*, and *ccl2* (*R. capsulatus*) products are suggested to form the heme lyase complex for heme attachment. Models combining the putative functions of the individual components of an integrated heme insertion process have been developed (51, 502, 816). The HelA/CycV proteins are the putative ATPases, and the HelBC/CycWZ proteins are the membrane components of the heme exporter specific for ligation (hence the acronym *hel*). Both CycK/Ccl1 and CycL/Ccl2 have putative heme-binding sites. The former proteins are membrane bound, whereas the latter are soluble or only membrane associated and may provide the heme to the proper heme lyase. An important component in the process is the *cycH* product. CycH is membrane bound, with a large periplasmic domain, and is assumed to direct the translocated apocytochrome to heme ligation (502, 686). Several genes for cyto-

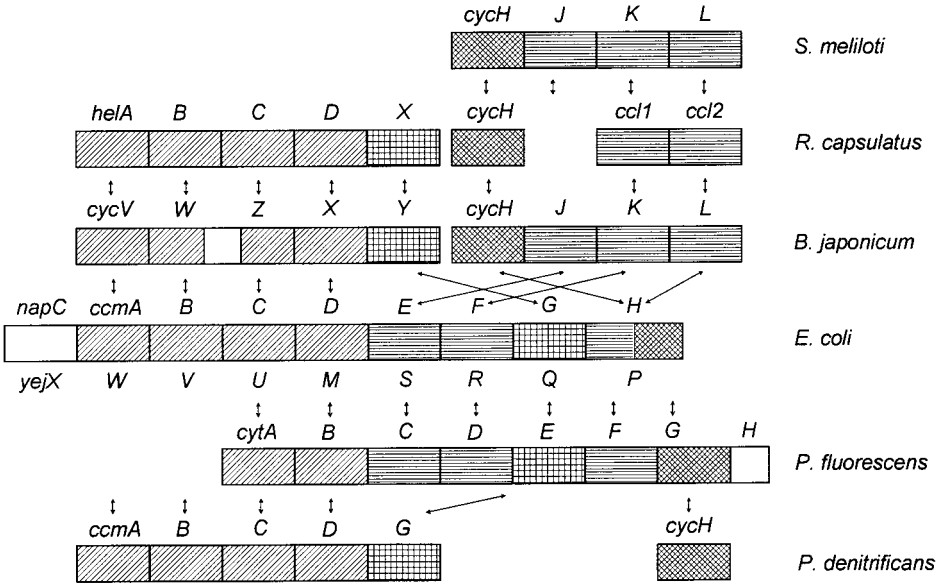

FIG. 17. Organization of gene clusters of denitrifiers and nitrate respirers encoding components of cytochrome *c* biogenesis. Groups of homologous genes (not drawn to scale) are indicated by identical patterns and are individually related by arrows. Open boxes have no homologs within the gene sets shown. *ccmH* of *E. coli* may be a fused gene combining the functions of *cycH* and *cycL*. Data are from *S. meliloti* (446), *Rhodobacter capsulatus* (50, 51, 502), *Bradyrhizobium japonicum* (668, 687), *E. coli* (303, 818), *P. fluorescens* (262), and *Paracoccus denitrificans* (630, 631).

chrome *c* biogenesis were also found in *Paracoccus denitrificans* (630, 631). Although some of them are homologous to *helABC*, their involvement in heme transport has been questioned (632).

Heme C is attached to apocytochrome via thioether linkages between the cysteines of the CxxCH sequence and the vinyl side chains of the heme. Site-directed mutagenesis of *cycA* from *Paracoccus denitrificans* has shown that both cysteine residues are required for heme attachment, suggesting the necessity of a disulfide intermediate (709). To open the disulfide bond thioredoxin-like proteins are required. The *helX* (*R. capsulatus*) and *cycY* (*tlpB*) (*B. japonicum*) gene products or similar proteins in other organisms may fill in this role (50, 165, 816).

The *ccmABCDEFGH* genes from the anaerobically expressed gene locus *aeg-46.5* of *E. coli* have been found to be homologous to the genes for cytochrome *c* biogenesis of *B. japonicum* and *R. capsulatus* (303, 818) (Fig. 17). The previous acronym *yej* was relinquished in favor of *ccm* to reflect the role of these genes for cytochrome *c* maturation. The *ccm* genes are located downstream of the *nap* genes, which encode the periplasmic nitrate-reducing system. They are controlled anaerobically from the *nap* promoter by FNR and the nitrate-regulated NarL or NarP factors, which provides a rationale for the differential ability of aerobic and anaerobic cells to synthesize *c*-type cytochromes (303). The *ccm*-encoded apparatus operates in a generalized mode since it does not discriminate between indigenous or foreign and periplasmic or membrane-bound cytochromes. However, certain components are duplicated and have a more specialized role for distinct cytochromes of the formate-dependent nitrite reduction pathway (302).

An unexpected finding is a linkage between excretion of the siderophore pyoverdine and the putative heme transporter (262). The *cytA* locus of *P. fluorescens* ATCC 17400 (homologous to *cycZ* of *B. japonicum* and *helC* of *R. capsulatus*, respectively) is part of a homologous gene cluster necessary for cytochrome *c* biogenesis (Fig. 17). Inactivation of *cytA* affects not only cytochrome *c* production but also the kinetics of pyoverdine excretion and the nature of excreted products. A *cytA* mutant is unable to grow under anaerobic conditions. The same locus in *P. fluorescens* 9906 affects copper homeostasis in addition to cytochrome *c* biogenesis (935).

NITRIC OXIDE-REDUCING SYSTEM

Reactivity of NO

Among the distinct chemical properties of NO is the presence of an unpaired electron, its reactivity with O_2 and $O_2^{\bullet -}$, charge neutrality, relatively long biological half-life, small Stokes' radius, and hydrophobicity, allowing facile intermembrane and transmembrane diffusion (241). NO in excess is toxic to bacteria, fungi, microbial parasites, tumor cells, and viruses. Although NO metabolism is innate to denitrifiers, the compound is toxic for this group of bacteria also. A knockout mutation for NO reductase is lethal, but the effect can be suppressed phenotypically by a further mutation in *nirS*, i.e., by inactivating the NO generator (99).

Understandably, the susceptibility of bacteria to NO has been studied, especially in pathogens. It is well established that murine macrophages produce NO. A clear manifestation of the bactericidal effect of NO is its synthesis via the inducible NO synthase as part of the macrophage antimicrobial defense system (reviewed, for example, in references 40 and 602). If NO synthase plays a role in host defense, its mutational loss or inactivation should produce strains with increased susceptibility to infection and this has been observed indeed (527, 898).

NO synthase is found throughout the *Eukarya*, generating NO as an inorganic messenger. In a single case so far, a *Nocardia* sp. was reported to produce NO by an NO synthase (146). Nocardiae are widely distributed in soil, from tropical to temperate regions, where they are believed to contribute to the turnover of plant material. They are also responsible for a wide range of animal diseases. A relation of NO synthase to infectivity has been suggested (145).

The toxicity of NO is a consequence of its reactivity with transition metal proteins and oxygen and its ability to form adducts with amines and thiols of varying stability (625). Heme Fe, nonheme Fe, and Cu-containing enzymes are the main targets where NO interferes with cellular processes. NO complexes in COX first with Cu_B and binds to the reduced heme A_3 during enzyme turnover causing inhibition (831). *Paracoccus denitrificans* cytochrome aa_3 might be inhibited by the same mechanism (124, 486). Another Cu-containing enzyme that is inhibited by NO is N_2O reductase (258). Mutations in *nor* genes lead to NO accumulation and affect N_2O reduction possibly by inactivating N_2O reductase (186). Iron-sulfur proteins are general targets for NO; the resulting Fe nitrosyls or breakdown products of Fe-S clusters are recognizable by EPR spectroscopy (333). Binding of NO to Fe-S centers is thought to be responsible for the inhibition of nitrogenase (377), which is of relevance for the diazotrophic denitrifiers.

The reaction of aqueous NO with oxygen comes to bear, when considering alternating aerobic and anaerobic life conditions of a denitrifier, or the continuous presence of oxygen for the microaerophilic and aerobic processes. NO is oxidized in aqueous solution by O_2 mostly to nitrite: $4NO + O_2 + 2H_2O \rightarrow 4H^+ + 4NO_2^-$. This simple equation involves a fairly complex chemistry with generation of intermediate, highly oxidizing species (288). Dinitrogen trioxide (N_2O_3) and nitrogen dioxide ($NO_2^{\bullet}$) are the reactive N- and S-nitrosating species produced during autoxidation of NO (289, 448).

An important aspect of NO reactivity is the formation of strongly oxidizing species by NO reacting with oxygen and the superoxide radical, $O_2^{\bullet -}$. Under aerobic conditions, NO will react with $O_2^{\bullet -}$ to give peroxynitrite ($ONOO^-$) in a nearly diffusion-controlled reaction (369). The high rate constant of 6.7×10^9 M^{-1} s^{-1} allows NO to compete effectively with superoxide dismutase for $O_2^{\bullet -}$. Peroxynitrite is a strongly oxidizing species, and effects attributed to NO are in part due to this compound (625). Peroxynitrite is much more toxic than NO for *E. coli* and kills the cells within 5 s of incubation (109). The same differential susceptibility toward NO and $ONOO^-$ is observed with *S. typhimurium* (188). The nitration of tyrosine by peroxynitrite can result in interference with regulatory functions of proteins that undergo phosphorylation or adenylylation. *E. coli* glutamine synthetase has been studied as a model for this type of action of peroxynitrite on vital cellular functions (73).

NO is mutagenic to bacterial DNA, where it causes C → T, AT → GC, and GC → AT transitions in *E. coli* because of its nitrosating and deaminating reactivity (565, 705, 910). Nitrosation of primary amines on nucleotides leads to deamination by the reaction of diazonium ions with water. NO inflicts also oxidative damage on DNA either by causing iron release from susceptible donors, resulting in Fenton-type chemistry, or through the mediation of peroxynitrite (201). The genotoxic effect of NO may also be of indirect nature by inhibiting DNA repair enzymes such as O^6-methylguanine-DNA methyltransferase or the zinc finger protein formamidopyridine-DNA glycolase (911). Overall, the action of NO on DNA is quite complex and extends to DNA strand breaks and cross-links (808). No type of DNA repair system is able to cope with this manifold reactivity, and finally cell damage or death results.

Bacterial Metabolism of NO

The formation and/or uptake of exogenous NO has been studied in a variety of bacteria including *P. fluorescens* (248), *P. stutzeri* (258, 723), *P. aeruginosa* (435, 886), *Flexibacter canadensis* (918), and *A. xylosoxidans* (541). Contrasting with the considerable number of physiological studies, the underlying biochemistry has been addressed only for *P. stutzeri* and *Paracoccus denitrificans* and to some extent for "*A. cycloclastes*." The amount of NO formed during denitrification depends on the organism, the culture conditions, and also the assay method, because the NO-forming system is susceptible to perturbations. Usually, a low pH favors production of NO over that of N_2O (886, 918). If a denitrifying culture of *P. stutzeri* is stripped by a carrier gas, it releases half to three-quarters of the supplied nitrate as NO (723, 953). Cells grown under oxygen limitation have a tendency to accumulate NO, an effect not observed with anaerobic cells (258, 292).

The K_m for NO uptake by whole cells of *P. stutzeri* has been estimated at 400 nM (953) but also substantially lower at 1.2 to 2.4 nM (675). A range of 0.5 to 6 nM is representative for *Azospirillum brasilense*, *P. aeruginosa*, and *P. fluorescens* (675). Irrespective of the considerable numerical divergence, the K_m obtained with whole cells is distinctly below that determined with the isolated enzyme. Substrate limitation or a structurally altered enzyme lacking the proper lipid environment may cause these higher in vitro values.

The concentration of extracellular NO during steady-state denitrification is in the low nanomolar range. The value of 50 nM for *P. stutzeri* obtained by gas stripping (953) agrees well with 20 to 30 nM obtained by a direct sensitive NO measurement (293). Denitrifying *P. aeruginosa*, supplied with nitrate, accumulates nitrite transiently and develops a steady-state NO concentration of 1 to 2 nM (435). Values ranging from 0.5 to 31 nM are representative of cell suspensions equivalent to 0.1 mg of protein per ml of "*A. cycloclastes*", *P. stutzeri* JM300, and *Paracoccus denitrificans* within 40 min of reaction time. The steady-state concentrations are higher with nitrite as the substrate (7 to 31 nM) than with nitrate (0.5 to 7 nM) (293). Given the free diffusivity of NO, these values should also give the range of intracellular NO concentration that a denitrifying cell will tolerate. NO toxicity, manifested as a loss of cell division and viability, is expected to occur around 1 mM and above, whereas protective measures of the cell against NO will have to cope with nano- to micromolar NO concentrations.

It is generally assumed that exogenous NO will not support cell growth because of its toxicity. NO-dependent bacterial growth has been found in strains of *Bacillus* that were isolated on nitrite by an anaerobic enrichment technique. Bacterial cell mass approximately doubled within 5 h in the presence of 10% NO, but the growth showed a long lag phase that could not be eliminated by subculturing (648). The successful approach for growing a bacterium on NO involves the presence of a low but continuous supply of NO and operation near the steady-state concentration of free NO during denitrification. To rigorously document bacterial growth on NO, the nonenzymatic conversion to N_2O and energy conservation via the latter substrate has to be excluded. Thus, growth of an NO-evolving strain of *P. aeruginosa* was possible only when N_2O reduction was not inhibited by acetylene, which means that NO by itself did not sustain the bacterial energy metabolism (886).

Breakdown of organic carbon and increase of biomass was shown recently to depend on NO. With only 0.5% NO in the feed gas, the cell mass of *Paracoccus denitrificans*, "*Pseudomonas denitrificans*," *Alcaligenes xylosoxidans* subsp. *denitrificans*, and *Thiobacillus denitrificans* (all induced anaerobically for denitrification with nitrate), increased at the expense of oxidizing a complex carbon source whereas the NO concentration fell to 0.01 to 0.02% in the exhaust gas (655).

Growth on NO as the sole electron acceptor should be possible since NO reduction is coupled to proton translocation (267, 744, 745, 886) and NO-dependent cell-free ATP synthesis

has been demonstrated (126). The conversion of NO to N_2O has a redox potential, E_0'(pH 7), of +1,177 mV and a free energy, $\Delta G^{\circ\prime}$, of −306.3 kJ/mol. It is energetically comparable to the respiration of N_2O to N_2 [E_0'(pH 7) = +1,352 mV; $\Delta G^{\circ\prime}$ = −339.5 kJ/mol], and both reactions are thermodynamically more favorable than the respiration of nitrate to nitrite (969). However, NO reductase is not considered to be a proton pump. Growth yield studies show that the overall reduction of nitrite to N_2O is energy conserving (464). Because of the difficulty in growing cells with NO, no comparable studies are available. Electron transfer to NO reductase proceeds through cytochrome bc_1 (126, 388), and energy conservation is associated with a dehydrogenase and/or the cytochrome bc_1 complex.

A small amount of NO sometimes originates from nitrate respiration but not denitrification (406, 435). The property to reduce NO is not restricted to denitrifiers. Strains of *Rhodobacter capsulatus* transform NO to N_2O at a significant rate of 10 to 35 nmol/min/mg protein (59). The methanotroph *Methylosinus trichosporium* (469), *E. coli*, *P. putida*, *Pseudomonas viridiflava*, and the cyanobacterium *Synechococcus* (*Anacystis*) *nidulans* (44) all consume NO under anoxic conditions without being able to denitrify.

A broad spectrum of mostly heterotrophic bacteria take up NO under oxic conditions and convert it to nitrate, whereas the anoxic reaction yields N_2O (44). The oxic reaction was discovered in soil samples depleted for nitrate (to make denitrification limiting) and has been studied more closely with a nondenitrifying isolate that is related to *P. fluorescens* and *P. putida* (468). NO oxidation by this isolate is not energy conserving. The reaction may have its distinct enzymology or may be due to a cooxidation effect.

NO is readily diffusible and will reach the cytoplasm or a neighboring cell within its biological half-life. NO easily penetrates proteins and lipid membranes to act at a site distant from its generation (501, 798, 862). In view of the numerous ways that NO can exert a cytotoxic or genotoxic effect on its own or in combination with other reactants, how do denitrifying bacteria cope? The control of nitrite reduction and NO reduction is coordinated to ensure removal of NO by NO reductase or, if this is not possible, to down-regulate nitrite reduction (see the section on regulation, below). The steady-state concentration of free NO during denitrification is maintained at a very low level. A major role in protecting an organism from NO resides in NO reductase itself. NO generation takes place in the periplasm, and the location of NO reductase in the inner membrane forms an effective sink to prevent NO from reaching the cytoplasm.

Induction of the nitrite-reducing system at only low oxygen tension will reduce the coincidence of NO and reactive oxygen species. Even aerobic denitrification is usually a microaerophilic process. A denitrifier that disposes over a periplasmic SOD can minimize the formation of peroxynitrite. The bacterial CuZnSOD is periplasmic and has been suggested to defend against exogenous $O_2^{\bullet -}$ or unknown sources of superoxide in this compartment (64, 474). CuZnSOD activity or the corresponding *sodC* gene have been detected in *Paracoccus denitrificans* (879) and *N. meningitidis* (474). CuZnSOD has been found in several pseudomonads, but only nondenitrifying species were tested (788).

Cytochrome *c′*, found in many denitrifiers, was suggested to function as an NO scavenger and to prevent the accumulation of NO to toxic levels (948, 949). Whether there is enough cytochrome *c′* in the cell to serve as an effective NO buffer is questionable, but such a protein may function together with other protective measures.

NO Reductase

Isolation and properties. Nitric oxide reductase was the last of the terminal oxidoreductases of denitrification whose molecular properties became known (329). The early demonstrations of cell-free NO reduction were not shortly followed by enzyme isolation, although NO reductase is not particularly unstable. The main difficulty in isolating this enzyme lies in its membrane-bound nature, which requires a detergent for solubilization. The manifold reactivity of NO with cellular components, not only metalloproteins, makes it more difficult to separate enzymatic from nonenzymatic processes and physiological from nonphysiological activities, and due care has to be taken (106, 655, 975). When NO consumption or formation is found as trace gas metabolism only, it may be a fortuitous property of a cellular component with no relation to denitrification.

NO reductase is not formed under aerobic culture conditions (420, 465, 676) and has to be purified from the O_2-limited or anaerobically grown cell. The appearance of NO reductase in *P. stutzeri* ZoBell is nearly concomitant with that of other enzymes of denitrification (465). Anaerobic formation of NO reductase is consistent with the presence of recognition motifs for FNR in the promoter region of the *norC* gene (23, 246, 968). Necessarily, in microaerophilic or aerobic denitrifiers, the expression NO reductase has to be derepressed at relatively high oxygen concentrations.

The first evidence for a membrane-bound NO reductase activity was obtained with *Alcaligenes faecalis* IAM 1015 (543). When sonicated cells were fractionated by differential centrifugation, about 80% of the activity resided in the membrane fraction whereas the minor part of cellular NO-reducing activity was attributed to cytochrome cd_1 in the soluble fraction (545). Distinct membrane-bound NO-reducing activities were described for *P. stutzeri* (742, 975, 982), *Rhodobacter sphaeroides* IL106 (850), *Halomonas halodenitrificans* (295), and *Paracoccus denitrificans* (123). Heme groups were occasionally found associated with NO-reducing fractions, but because of the lack of purified enzyme, no unequivocal evidence could be provided for the cytochrome nature of the enzyme. The lingering question was for many years whether there is a discrete NO reductase at all or whether the NO reduction is the accidental property of nitrite reductase or of a component of the respiratory chain. This question was finally resolved when genetic evidence was provided for a separate NO-reducing activity and proved that cytochrome cd_1 or CuNIR does not represent the denitrifying NO reductase (99, 939, 972).

The still popular assay system for cell-free NO reduction with asc-reduced PMS originated from Mori's laboratory (543, 582). Making use of this assay, the NO reductase from the membrane of *P. stutzeri* was solubilized and purified sufficiently to show its nature as a cytochrome *bc* complex (Fig. 18) (976). This opened the way to a reproducible method of purification and the elucidation of basic properties of the enzyme (329, 441). A homogenous NO reductase was obtained from *P. stutzeri* by using Triton X-100 as the solubilizing detergent, chromatography on DEAE cellulose and hydroxylapatite, and gel filtration. A similar procedure worked successfully for *Paracoccus denitrificans* NCIB 8944 (125), and modified simpler methods were described for two other strains of *Paracoccus* (200, 260). The CuNIR-synthesizing "*A. cycloclastes*" has an NO reductase with properties not different from those of the cytochrome cd_1-containing denitrifiers (420).

Dodecyl maltoside (125, 279, 420), octyl glucoside (200), and sucrose monocaprate (260) are effective detergents for enzyme isolation. Triton X-100 decreases the activity of NO reductase,

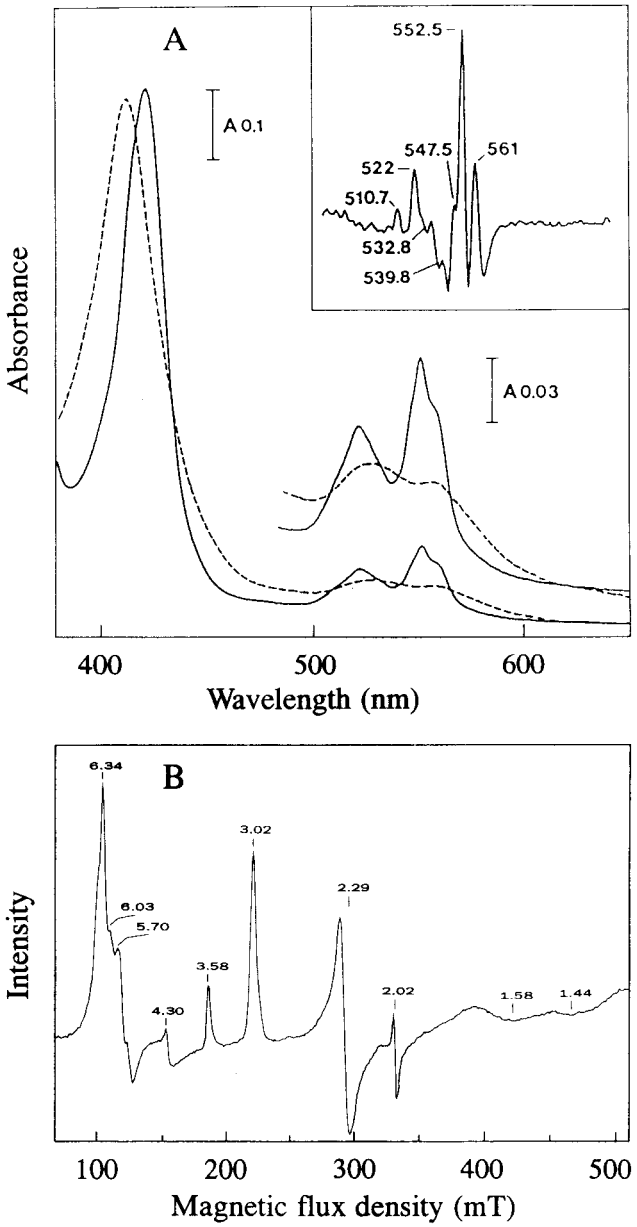

FIG. 18. Electronic absorption spectra (A) and EPR spectrum (B) of the NO reductase complex of *P. stutzeri* ZoBell. (A) Dotted line, enzyme as isolated; solid line, enzyme after the addition of dithionite. (Insert) Second-derivative spectrum of the reduced-minus-oxidized enzyme form. Reproduced from reference 329. (B) EPR spectrum of the reductase as isolated in 20 mM Tris-HCl (pH 8.5)–0.1% Triton X-100. Recording was done at 9.31-GHz microwave frequency, 100-kHz modulation frequency, 1.0-mT modulation amplitude, 81.9-ms time constant, 168-s scan time (22 scans), 2.0-mW microwave power, and 9 K. Reproduced with permission from reference 441.

particularly in the critical step of solubilization (329, 420). Incubation of crude extracts from several denitrifiers for several hours with 5 to 7% Triton X-100 even completely abolishes NO-reducing activity (742). However, inclusion of phospholipids or a detergent, for example octyl thioglucopyranoside, in the assay mixture reconstitutes a considerable part of the enzyme activity. Delipidation by Triton X-100 may occur when the enzyme is removed from the membrane and may cause the decrease in activity. The stability of NO reductase is maintained best in dodecyl maltoside or laurylpropanediol-3-phosphorylcholine ether. It is less stable in octyl glucoside or its thioderivative, although these detergents are good enhancers of activity (200, 441).

The requirement for a phospholipid has been studied with the *P. stutzeri* enzyme (441). Diphosphatidylglycerol shows the most pronounced effect in activating NO reductase. The major lipid of the purified enzyme complex is phosphatidylethanolamine, which is the most abundant phospholipid of *P. stutzeri* (621) and therefore may not be a specific component of the enzyme. Free fatty acids are inhibitory for activity. The chain length and the degree of saturation affect the strength of inhibition. The environment of the high-spin heme of NO reductase (probably part of the active site) is sensitive to hydrophobic interactions as seen in alterations in the EPR spectrum in different detergents.

Bacterial nitric oxide reductases are complexes of at least two subunits of 17 and 53 kDa (Table 7). The mass obtained by SDS-electrophoresis coincides with that of the nucleotide sequence-derived value only for the small subunit, NorC. The large subunit, NorB, is a highly hydrophobic *b*-type cytochrome that deviates notably in its apparent mass of 34 to 38 kDa from the real value. Stoichiometries of one or two small subunits per large subunit have been proposed. The mass of the complex found by gel filtration in detergent is higher than that of a dimeric complex (also including the mass contribution from the detergent micelle) and makes aggregates of protomers likely (Table 7).

The absorbance spectrum of NO reductase shows the spectral features of heme C and heme B (Fig. 18A). In the reduced form, the isolated NorB subunit has absorption maxima at 428, 531, and 560 nm. On electrophoretic separation of the reductase complex, NorC retains heme C (441). The isolated, reduced NorC subunit has absorption maxima at 418, 523, and 551.5 nm. NorC is a membrane-bound monoheme *c*-type cytochrome. A single sequence motif, CxxCH, for covalent heme C attachment is located in the N-terminal domain following the transmembrane helix for anchoring the protein in the membrane (23, 186, 968).

Preliminary genetic evidence indicates that NO reductase is subject to activity regulation (968). *nir* mutants of *P. stutzeri* have a drastically lowered expression level of the enzyme, yet the cellular activity of NO reduction is comparable to that of the wild type. The in vitro activity corresponds to the extant amount of enzyme, which means that a large part of the reductase found in the wild type is down-regulated in its activity.

Structural model. The strong hydrophobicity of the cytochrome *b* subunit of NO reductase finds its explanation in 12 hydrophobic segments of the primary structure. Predictive algorithms for the topology of membrane proteins arrange them in the form of alternating transmembrane segments (966, 968). An important development was the finding that NorB is similar to the subunit I of the heme-copper oxidase family of terminal oxidoreductases. A sequence alignment suggested the correspondence of the membrane-spanning regions of the two proteins (714, 863), although the overall conservation of primary structure falls with 18 to 25% in the twilight zone of uncertain sequence relatedness (978). The original conclusion, based on the primary structure of *P. stutzeri* NO reductase, was confirmed by the later determination of additional NorB sequences (23, 163, 186). Precise information about the transmembrane helix arrangement of a heme-copper oxidase comes from the crystal structure of *Paracoccus denitrificans* COX (395). The combination of the NorB primary structures and the crystallographic data provides a solid basis for comparison and suggests a model for NO reductase derived from the COX

TABLE 7. Properties of NO reductases (EC 1.7.99.7)

Property	Value for:		
	Pseudomonas stutzeri	*Paracoccus denitrificans*	"*Achromobacter cycloclastes*"
Mol mass[a] (kDa)	180 (Triton X-100)	160–170 (dodecyl maltoside)	
Subunit mol mass (kDa)	17, 38 [17.3, 53][b]	17.5, 38 [17, 52.3][b]	17.5, 38
Prosthetic groups	Heme C and B, nonheme Fe	Heme C and B, nonheme Fe	Heme C and B
UV-visible maxima (nm)			
As isolated	411.5, 537, 558sh[c]	276–277, 411, 525, 556.5	412, 525, 551
Reduced form	420.5, 522.5, 552.5, 560sh	420, 522.5, 551.5, 562sh	420, 523, 551, 559.5sh, 600
ε (mM^{-1} cm^{-1}, Soret band)	310–350		112
EPR parameters (*g* values)			
Low-spin hemes	3.54; 2.97, 2.25, 1.43	3.53; 2.99, 2.28, 1.46	
High-spin heme	5.7–6.3	6.2–5.8	
Nonheme Fe	2.01–2.02?	2.009?	
Sp act (μmol of NO · min^{-1} · mg^{-1})	60 (with phospholipid)	10–22	4–5
pH optimum	4.8	5	5.5
Electron donor	PMS-asc	TMPD[d]-asc, cytochrome *c*	PMS-asc
K_m for NO (nM)	1.2–2.4[e]	250[f]	
K_m for PMS (μM)	2		
K_m for O_2 (μM)	No activity	8.4[f]	No activity
Reference(s)	143, 329, 441, 968	125, 200, 279	420

[a] By gel filtration in the indicated detergent.
[b] Sequence-derived values from references 186 and 968.
[c] sh, shoulder.
[d] TMPD, *N*,*N*,*N*′,N′-tetramethyl-*p*-phenylenediamine.
[e] Value from reference 675.
[f] Value from reference 260.

structure (437, 978). The metal-binding ligands of the subunit I of COX have positionally corresponding residues in NO reductase within an overall conserved array of membrane-spanning segments.

On elaborating the structural similarity between NorB and COX, one finds the α helices of NorB are nearly superimposable on that of COX I (Fig. 19A). Modeling the atomic coordinates of NorB on the basis of COX I results, for NorB, in a compact hydrophobic molecule with limited polar surface areas exposed on either side of the membrane (437). When viewed from the periplasmic side, the 12 helices are arranged in three semicircles in a sequential, counterclockwise manner beginning with helix 11. One arc, formed by helices 11, 12, 1, and 2, envelops heme B, which is proposed to be bound by His60 and His349 from helices 2 and 10, respectively. Another arc, formed by helices 7, 8, 9, and 10, forms the second heme pocket. Heme *b* is proposed to be bound by His347 from helix 10. In following the COX I arrangement for NorB, it places the conserved residues His207 (helix 6) and His258 and His259 (helix 7) in a position which is proposed to represent a third metal center in NorB, equivalent to Cu_B of COX I (978).

Chemical analysis of NO reductase yields more Fe than is accounted for by the heme content alone (200, 329). This extra Fe is not an iron-sulfur species, since no corresponding signal exists in the EPR spectrum (Fig. 18B). The extra Fe is therefore presumed to be a nonheme Fe species at the metal center formed by the histidines from helices 6 and 7 (Fig. 19B). The stoichiometry of nonheme Fe to heme B and heme C Fe in a minimal composition of the reductase complex of one cytochrome *b* and cytochrome *c* each is 1:2:1. Analytical data that satisfy such a stoichimetry have been provided for the *Paracoccus* reductase (279). NO reductase does not contain a significant amount of Cu to form a bimetallic center as in COX.

SDS-electrophoresis shows further protein species in the three purified NO reductases; that is, could there be more than two subunits in the NO reductase complex? A 45-kDa band seen with the *P. stutzeri* enzyme dissociates on repeated electrophoresis into 17- and 38-kDa subunits; on heating, the isolated 38-kDa subunit forms an aggregate of 74 to 78 kDa. The presence of the large subunit is proven by cross-reactivity with an anti-NorB antiserum and shows that the higher-mass species do not represent different peptides (441). A preparation of *P. denitrificans* NO reductase has one additional 77-kDa band as an aggregation of the 38- and 17.5-kDa subunits (200). The organization of the structural genes for NO reductase of *P. stutzeri*, where the *norCB* genes form an operon with a single transcript of 2.2 kb that covers both genes, suggests a two-subunit core complex (968). NO reductase purified in dodecyl maltoside consists of only the cytochrome *c* and cytochrome *b* subunits (279). A minimal composition of two subunits is thus clearly sufficient for an active enzyme.

The idea of an additional peptide in NO reductase in situ originates from the deduced *orf2* gene product, which shows similarity to subunit III of COX. First described for *P. aeruginosa* (21), an *orf2* homolog was found in *Paracoccus denitrificans* (there termed *norE*) (186) and in *P. stutzeri* (*orf175*) (283) (Fig. 2). The COX from *Paracoccus denitrificans* had initially been purified as a two-component enzyme but was later found to have a third subunit (314, 523). Although COX III appears to affect energy conservation (920), it is not essential (315) and is believed to play a role in oxidase assembly (313). The crystal structure of the oxidase complex has proven the presence of a fourth, very small subunit consisting of a single transmembrane helix (395). The primary structure of this fourth oxidase subunit (913) has no equivalent in any derived protein of the denitrification gene clusters.

The *orf2* product and homologs are hypothetically five-span membrane proteins (17.7 to 19.5 kDa) that have ≈30% sequence identity to membrane helices 3 through 7 of COX III whereas helices 1 and 2 are absent. The crystal structure of COX III shows the proximity of helix 3 to helix 6 via a histidine residue in helix 6 that is hydrogen bonded to the dicyclohexyl carbodiimide-binding residue Glu97 of helix 3 (395). Both residues are conserved in the ORF2 protein and homologs and

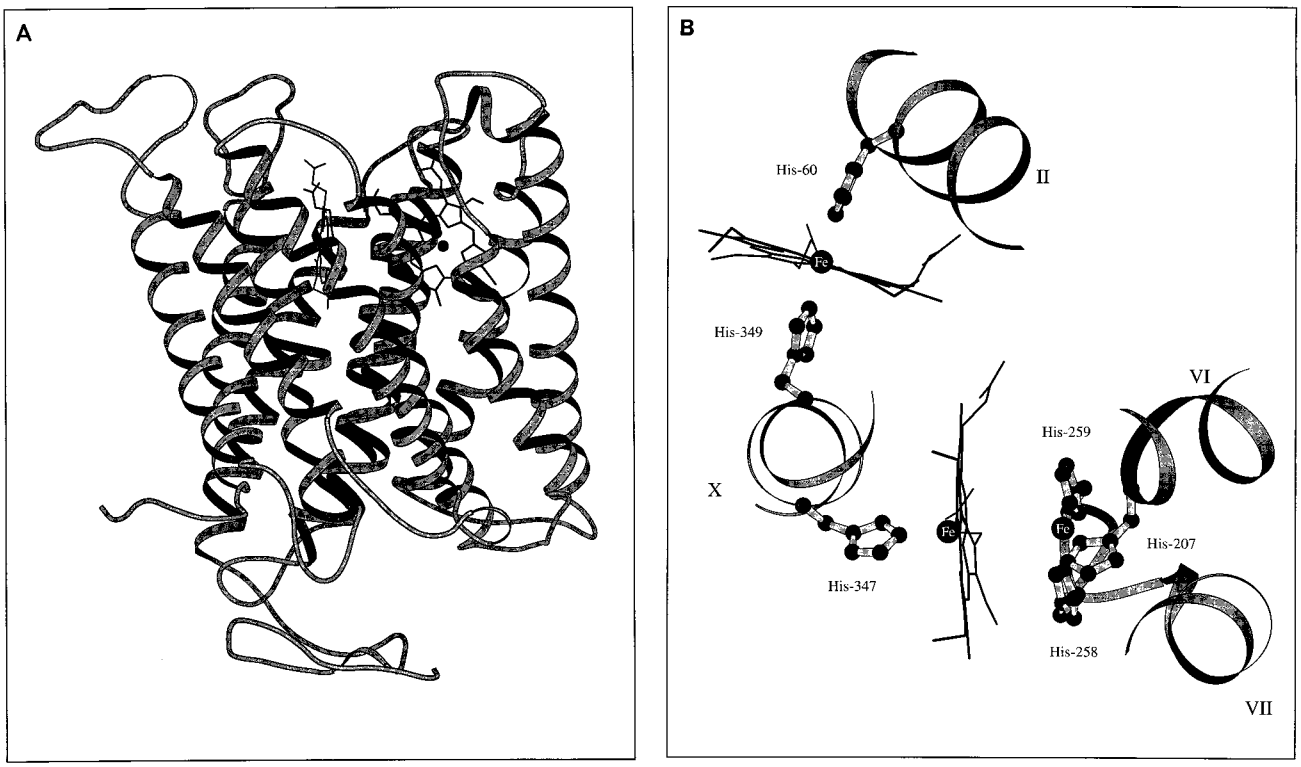

FIG. 19. Model for the membrane topology of the cytochrome *b* subunit of bacterial NO reductase. (A) The model is based on the amino acid sequence derived from the *norB* sequence of *P. stutzeri* (968). The atomic coordinates of the crystal structure of *Paracoccus denitrificans* COX I (395) were used to individually match each amino acid for NorB. The positions of two *b*-type hemes and the nonheme Fe are shown. The periplasmic side is on top. (B) Model for the geometry of the metal centers of NorB. The three Fe sites comprise the low-spin heme B coordinated by His60 and His349, the high-spin heme B coordinated by His347, and the nonheme Fe coordinated by three histidines and possibly water ligands (not shown). The binuclear site is proposed to represent the active site of the enzyme. Courtesy of H. Michel and A. Kannt.

suggest an arrangement of the transmembrane segments similar to COX III (978). *orf2* is arranged in *P. aeruginosa* and *P. stutzeri* as part of a putative operon, *nirQ* → *orf2* → *orf3*, upstream of *nirS* and in the opposite transcriptional direction (Fig. 2). Mutational inactivation of *nirQ* results in an inactive NO reductase, and deletion of *nirQ* together with *orf2* and *orf3* abolishes denitrifying growth. Both defects are complemented in *trans* by *nirQ* alone, which indicates nonessentiality for both the *orf2* and the *orf3* products (24, 431).

The gene arrangement downstream of *norCB* in *P. denitrificans* is *norQDEF* (186) (Fig. 2). *norQ* is homologous to *nirQ*; *norD* encodes a protein of 69.7 kDa and is homologous to ORFs immediately downstream of *norB* in *P. aeruginosa* and *P. stutzeri* (24, 283). Inactivation of either *norQ* or *norD* affects nitrite, NO, and N_2O reduction simultaneously (186). The precise functions encoded by these genes remain to be established. The *norF* product, which has no equivalent in the pseudomonads, is hypothesized to present, together with NorE, a third and fourth subunit of NO reductase (186). Evidence for this will require demonstration of these gene products as part of the isolated enzyme, which so far has not been achieved by using different detergents and purification methods. The inactivation of *norE* and *norF* affects the activity but not the expression level of NO reductase.

Mechanistic aspects. Nitric oxide reductase catalyzes the reduction of NO to N_2O: $2NO + 2H^+ + 2e^- \rightarrow N_2O + H_2O$ [E_0'(pH 7.0) = +1.18 V; $\Delta G^{\circ\prime}$ = −306.3 kJ/mol]. Since the reaction involves dimerization of a mononitrogen species to form the N—N bond, the overall reaction requires two electrons. The cytochrome bc_1 complex and cytochrome c_2 are optional components in the electron transfer to NO in photodenitrifiers (59, 716). This suggests branching of the electron transfer pathway at the ubiquinol level and existence of alternative electron donors for NO reductase within the same organism. Solubilized NO reductase activity is dependent on the concentration of cytochrome c_2. The interaction with the electron donor is thought to be electrostatic, since the activity decreases rapidly with increasing ionic strength (388). Mutational inactivation of cytochrome c_{550} of *P. denitrificans* PD1222 does not affect denitrification, but NO reduction of a *cycA* mutant becomes sensitive to DDC (30% inhibition), which indicates that azurin may be an electron donor for the reductase (587).

The reasonable port of entry for electrons to NO reductase is the cytochrome *c* (NorC) subunit. A view of electron flow from NorC to NorB is supported from the midpoint potentials for hemes *c* and *b* of +280 and +322 mV, respectively (441). NorC is a membrane-bound bitopic cytochrome with the N terminus oriented toward the cytoplasm and a large heme C-binding domain of ≈120 amino acids residing in the periplasm. This orientation is deduced from an enzymatically active translational PhoA fusion at residue Leu67 immediately behind the heme attachment site, 61-CIGCH-65, of NorC (437). The heme C domain can interact with a periplasmic cytochrome or azurin as the electron donor and can supply electrons to the membrane-bound NorB. As deduced from the model, the heme groups of NorB are somewhat closer to the

periplasmic than the cytoplasmic face of the membrane (Fig. 19A).

The optical features of NO reductase indicate the presence of low-spin heme B and heme C and of a high-spin heme B in the absorbance properties of the CO-reacted material (260). The composite EPR spectrum is interpreted to arise from high-spin heme ($g \approx 6$, 1.58, and 1.44), a low-spin heme Fe ($g \approx 3.02$, 2.29, and 2.02), and a signal perhaps representing an interacting Fe species ($g = 3.58$) (441). On addition of NO to the reductase, the formation of a heme nitrosyl complex is observed. The MCD spectrum of NO reductase shows bands originating from a low-spin heme C (with histidine and methionine coordination attributed to NorC) and from a low-spin heme B (with *bis*-histidine coordination) and a high-spin heme B, both attributed to NorB (143). The heme B-nonheme Fe binuclear site of NO reductase (Fig. 19B) is proposed to represent the catalytic site homologous to the heme A_3-Cu_B bimetallic center of COX I. This is supported by site-directed mutagenesis of His258, which abolishes the enzymatic activity of NO reductase (437).

A synthetic organometallo complex, combining heme-coordinated Fe via an oxo bridge to a nonheme Fe, mimics the binuclear Fe site of NorB (536). The compound is similar to structural models for the heme Fe-Cu_B site of oxidases. The nonheme Fe site in NorB is likely to involve further ligands in addition to the three histidines to provide a suitable coordination sphere for Fe. The NorB model yields no discrete clues to the nature of the additional ligands (except of water molecules), since no carboxylate or hydroxy amino acid is in sufficient proximity (Fig. 19B) (437).

The reaction mechanism for the denitrifying NO reductase has to account for the binuclear heme/nonheme Fe center. Binuclear, redox-active sites that reduce NO to N_2O are found in COX (108), ribonucleotide reductase (322), and hemocyanin (875). Unexpectedly, since this does not seem to be a general property of NO reductase, the enzyme from *P. denitrificans* GB17 reduces O_2 (260). This is the first report of such an activity. It implies that the chemistry at the binuclear centers of NO reductase and COX is similar. Note also that NO^- as a putative reaction intermediate for N_2O formation is isoelectronic with O_2.

Proximity, provided by a binuclear site, appears to be a factor in N—N bond formation. For N_2O formation by ribonucleotide reductase, the *syn* form of two NO molecules bound at the same side of the binuclear Fe center is thought to be more competent than the *anti* form. The binding of two molecules per Cu pair is suggested for the oxidation of deoxyhemocyanin by NO (875). In CuZnSOD, only the Cu, not the Zn, is reactive with NO. NO^- but not N_2O formation has been reported for the enzyme (594).

Prior to the current evidence for a binuclear site in NO reductase, two different mechanisms of NO reduction were suggested whose basic features are still tenable, even though both were based on mononuclear heme and nonheme centers. Hollocher et al. proposed a one-electron reduction of NO to nitroxyl anion (NO^-) (270, 291, 354). Reduction of NO is thought to occur at a heme group of the reductase, with N_2O formation proceeding nonenzymatically by dimerization of the nitroxyl and dehydration via the presumed intermediacy of hyponitrite: $NO^- + H^+ \leftrightarrow HNO$; $2HNO \rightarrow [HO—N{=}N—OH] \rightarrow N_2O + H_2O$. HNO is a weak acid ($pK_a = 4.7$ [296]). At physiological pH NO^- is the predominant form that yields N_2O on protonation. The trapping of a reactive NO species by thiol is taken as supportive evidence for the formation of NO^- (841). The reaction has a chemical precedent in the stepwise reduction of nitrite to NO and N_2O in acidic $FeSO_4$ (89, 90).

If electrons flow in NorB from the high-spin heme B to the low-spin heme B of the binuclear center, as implied in the model (Fig. 19B), a reduced heme B is generated during the catalytic cycle at the substrate-binding site. Ferrous heme complexes have an extremely high affinity for NO (695, 753). Averill et al. have therefore argued that the redox potential of the heme Fe^{2+}-NO^0/Fe^{2+}-NO^- couple is, at -0.9 V, too negative to be accessible by a physiological reductant. The preferred reaction of heme Fe^{2+}-NO^0 would be the oxidation to heme Fe^{2+}-NO^+. As an alternative the formation of a nonheme Fe dinitrosyl complex at the active site has been suggested for N—N bond formation (940). A dinitrosyl complex is attractive, since it brings two NO molecules close together, which would not be possible at a monoheme site. Of relevance for the reactivity of nonheme Fe toward NO is the ferrous ascorbic acid complex, which under physiological conditions readily produces N_2O from NO (975). This reaction can also provide a potential sink mechanism for NO in higher organisms.

Heme centers in general cannot be ruled out from catalyzing NO reduction. The ammonifying multiheme nitrite reductases from *E. coli*, *Wolinella succinogenes*, and *Desulfovibrio desulfuricans* have no additional nonheme iron but reduce NO to N_2O with PMS-asc at rates even above that of NO reductase (156). Another example of an NO-reducing heme protein is the fungal NO reductase, cytochrome P-450nor, described in the following section. The enzyme is a monoheme protein with a redox potential that is unusually low for a member of the P-450 family.

FUNGAL DENITRIFICATION

For more than a century, denitrification has been considered a prokaryotic process and it was assumed that the mass transfer of fixed nitrogen back to N_2 exclusively proceeds through bacterial activities. Nevertheless, over the years, fungi have had an intermittent record of denitrification. N_2O formation was found in the genera *Fusarium*, *Giberella*, *Trichoderma*, *Cylindrocarpon*, *Chaetomium*, *Penicillium*, *Aspergillus*, and *Hansenula* and in a considerable number of other members of the Fungi Imperfecti, filamentous fungi, and yeasts (83, 85, 111, 758, 947). The early reports were equivocal with respect to an inhibitory or stimulatory effect of oxygen, association of the reaction with nitrate assimilation, and the use of nitrate or nitrite as the active substrate. The singular report of dissimilatory nitrate reduction in a ciliate attracted attention but was not pursued further (247).

Whole-cell activity of N_2O formation is strongest in outgrown fungal cultures but still is very low. Rates reported in different studies are difficult to compare because of differences in experimental design and the lack of a common reference parameter. Estimates from published figures give maximum rates of approximately 0.12 and 0.44 nmol of N_2O per ml of culture volume per day for *Hansenula* sp. (83) and *Giberella fujikuroi* (758), respectively. Shoun et al. investigated a large number of denitrifying fungi and found nitrite to be the better substrate for N_2O formation than nitrate in the absence of oxygen. N_2O is uniformly labeled from either [^{15}N]nitrate or [^{15}N]nitrite, clearly revealing its provenance (758). Occasionally, NO is a minor product in addition to N_2O; *Talaromyces flavus*, however, preferentially forms NO. Dinitrogen is formed by *Fusarium solani*, *Cylindrocarpon tonkinense*, and several other fungi but not by *Fusarium oxysporum*. The fungal system acts in the presence of nitrite also on other nitrogen-containing compounds such as azide and salicylhydroxamic acid to yield

N_2 and N_2O, where part of the latter is found as a $^{14}N^{15}N$ mixed species (809). A nitrosating mechanism is invoked for the mixed-species formation.

The molecular entity responsible for fungal denitrification remained obscure until a microsomal and a soluble cytochrome P-450 were found in *F. oxysporum*. The soluble variant of this cytochrome is increased under anaerobiosis and in the presence of nitrate or nitrite (759). N_2O is the product of denitrification by *F. oxysporum*, indicating the absence of N_2O reductase. *F. oxysporum* and *C. tonkinense* are currently the two best-studied representatives of fungal denitrification. The former was shown to have reductases for nitrate, nitrite, and NO, and the latter had those for nitrite and NO. Further studies are required in cases where the initial observation had indicated the formation of N_2 to prove whether certain species use a complete denitrification pathway and, if so, to clarify the nature of the N_2O reductase. Of the two cytochrome P-450 activities initially identified, the membrane-bound microsomal enzyme is a flavoheme protein with hydroxylase activity for fatty acids (600, 601).

The sharing of fungal denitrification in energy conservation is still under investigation. NADH is the direct reductant of the enzyme without involving a membrane-bound electron transfer chain as in bacterial denitrification: $2NO + NADH + H^+ \rightarrow N_2O + H_2O + NAD^+$. Since *F. oxysporum* uses an NO-generating nitrite reductase, detoxication was thought the preferred function for this enzyme (597, 759). On the other hand, growth is slightly better when cells are cultured in the presence of nitrate or nitrite (759, 854). Mitochondria of *F. oxysporum* were found to be associated with a membrane-bound nitrate reductase which can be solubilized by desoxycholate. With malate and pyruvate as substrates, nitrate reduction proceeds concomitantly with ATP formation (460). The nitrite reductase of *C. tonkinense* also resides in the mitochondrion, where it is in the intermembrane space as deduced from its soluble nature and from fluorescence labeling. This can be seen as an evolutionary parallel to the periplasmic location of CuNIR in bacteria. Nitrite-to-NO reduction is reported to yield ATP with succinate or malate and pyruvate.

Dissimilatory NO Reductase

The enzyme responsible for the conversion of NO to N_2O is a cytochrome P-450 labeled with the epithet "nor" to distinguish it from other members of the cytochrome P-450 family (597, 759). P-450nor is a distinct member of the family of eukaryotic P-450 proteins since it does not catalyze a monooxygenase reaction in spite of having structural similarity to P-450 oxygenases. The enzyme is a soluble monoprotoheme cytochrome with a mass of 44,371 Da (sequence-derived value). The protein as isolated has absorption bands at 414 (Soret), 532, and 566 nm; weak features are present at 390 and 643 nm. On reduction, the Soret peak shifts to 408 nm. In the ferrous CO-complexed enzyme form, the Soret band is at 447 nm, qualifying the protein as a cytochrome P-450.

NO is stoichiometrically converted to N_2O at about 300 μmol of NO min^{-1} mg^{-1}, which is five times the rate of the bacterial NO reductase and far greater than in vivo rates for fungi. Possibly, nitrate or nitrite reduction is rate limiting in whole cells. The K_m for NO is 0.1 mM, much higher than that for the bacterial enzyme. Crystals of the protein diffract to 2.5 Å, suitable for structure determination (596). P-450nor receives electrons directly from the reduced pyridine nucleotide. No auxiliary flavoprotein or iron-sulfur protein and no nonproteinaceous or artificial carrier is required for the electron transfer from NADH. To this end, the cytochrome can be expressed in *E. coli* in a soluble form that is active with NADH and needs no further cofactor (616). Overall, two electrons are required for the reaction $NO \rightarrow N_2O$, yet the reaction is unusual in the direct coupling of the hydride-donating NADH (two electrons) to a cytochrome without mediation by a flavin.

The reaction mechanism of this enzyme is thought to be different from that discussed for the bacterial NO reductase. The resting state of P-450nor exhibits a mixture of low-spin and high-spin Fe in the EPR spectrum (757). The redox potential of P-450nor, at −307 mV, is exceptionally low in comparison with that of other P-450 cytochromes. Electron transfer from NADH occurs on binding of NO to the ferric state of the enzyme, which is thought to produce a conformational change to a state competent for electron transfer. The ferrous NO complex detected by EPR spectroscopy is unstable and changes within minutes to a denatured enzyme form. Reduction of P-450 by dithionite yields a P-450($Fe^{2+}NO$) species whose spectral properties (maxima at 434 and 558 nm) are different from those of an NADH-reduced P-450 species (maxima at 444 and 544 nm). It is assumed that a reactive P-450 $(Fe^{3+}NO)^{2-}H^+$ species is formed which releases N_2O by a reaction with a second molecule of NO (756).

The primary structure of P-450nor was deduced from a cDNA clone carrying the structural gene CYS55 (456). It shows a low sequence similarity (average of 22%) to other members of the P-450 family, including as sources mammals, insects, plants, fungi, and other bacteria. It has only about 25% identity to *P. putida* $P\text{-}450_{cam}$; the relationship with $P\text{-}450_{cam}$ and $P\text{-}450_{SU2}$ from streptomycetes is significantly closer (34 to 40% identity). CYS55 shows the features of an eukaryotic gene with seven introns of 49 to 55 bp, a poly(A) tail, and a typical poly(A) tail-addition signal. The transcription of CYS55 responds to nitrite and nitrate; the promoter shows putative binding motifs for the bacterial response regulator NarL and an FNR factor (828). Their functionality, however, is not proven experimentally, and the presence of those motifs may be fortuitous. Isoforms of cytochrome P-450nor have been isolated from *C. tonkinense* (487, 829) and *F. oxysporum* (595). These enzymes have the same catalytic mechanism but exhibit different electron acceptor specificity toward NAD(P)H or show distinct N-terminal processing.

Dissimilatory Nitrite Reductase

In view of the high NO-reducing activity of *F. oxysporum*, the question arose which enzyme provides this substrate. Nitrite reductases of fungi are assimilatory-type siroheme-containing proteins that form ammonia, and no dissimilatory enzyme has been isolated before. The search for the NO-generating enzyme turned up a Cu-containing enzyme with properties of the bacterial blue CuNIR (461). The enzyme is a dimer in gel filtration with a subunit mass of 41.8 kDa in SDS-electrophoresis, reminiscent of other blue bacterial nitrite reductases which, initially described as dimers, were later found to be trimers. The enzyme is soluble but associated with the mitochondrion. EPR spectra show the presence of both type 1 and type 2 Cu. *F. oxysporum* also synthesizes a low-molecular-mass azurin-like protein (15 kDa) that acts as an electron donor to the nitrite reductase. A *Fusarium* cytochrome c_{549} and likewise a yeast cytochrome *c* act as electron donors. Coupled together in vitro, the fungal CuNIR and NO reductase catalyze the formation of N_2O from nitrite (461).

Fungal denitrification may be of a more general significance since it is not limited to a singular example. Fungi are suspected to contribute to atmospheric N_2O and the greenhouse effect. Horizontal gene transfer between fungi and bacteria has

been invoked as the rationale for the occurrence of bacterial regulatory elements in the fungal gene. The fact that fungal denitrification shares certain elements with the bacterial process hints at common roots and is reinforced by the existence of a bacterial-type nitrite reductase.

RESPIRATION OF NITROUS OXIDE

N_2O Reduction, an Autonomous Respiratory Process

The conversion of N_2O to N_2 is the last step of a complete denitrification pathway and represents a respiratory process in its own right. Many denitrifying bacteria grow at the expense of N_2O as the sole electron acceptor for the oxidation of organic compounds, certainly a reflection of the role of N_2O as an obligatory free intermediate. The property had been used as a screening method for the enrichment of N_2O-utilizing *P. stutzeri* from Senegal soil (649) or for the isolation of mutants affected in N_2O respiration (971). Aquifer microorganisms mineralize benzene and alkylbenzenes at the expense of N_2O, which indicates that the process is of significance in natural habitats (374). The plasmid-encoded nature of N_2O respiration in *R. eutropha* and *S. meliloti* further underlines the separate status of this respiratory process. Involvement of the cytochrome bc_1 complex in N_2O reduction was demonstrated for *P. denitrificans* (91, 640), *Rhodobacter sphaeroides* (387), and *Rhodobacter capsulatus* (387, 683). Electron transfer through cytochrome bc_1 is accompanied by proton transfer across the membrane. N_2O reduction is therefore energy conserving even though N_2O reductase is a soluble enzyme (494, 681, 853).

A truncated version of denitrification results when N_2O respiration is lacking and the process terminates with N_2O. It is likely that in all those cases the genetic information for N_2O reductase is absent. The truncated denitrification variant was initially described for "*Corynebacterium nephridii*" (318, 677), *P. chlororaphis*, and a strain of *P. fluorescens* (299); more recently N_2O was found as the end product in most carboxidotrophic bacteria (257), *Roseobacter denitrificans* (211, 750), *Thauera aromatica* (12), *Thauera selenatis* (528), *Azoarcus evansii* (12), and *Bacillus halodenitrificans* (197). The loss of N_2O reduction can be induced by mutation (971), which mimics the natural variant of truncated denitrification. The absence of N_2O reduction does not affect the reaction steps from nitrate to N_2O. It also has to be noted that sometimes a complete denitrifier that uses all the enzymes does not reduce exogenous N_2O (110, 286, 777), which is thought to be due to loss of the enzyme (779).

Just as nitrate respiration is not coupled obligatorily to denitrification, bacteria that respire N_2O without being denitrifiers are known (46, 553, 950). *Wolinella* (formerly *Vibrio*) *succinogens* is the first strain that was shown to grow on N_2O. The bacterium oxidizes formate by reducing nitrate to nitrite, nitrite to ammonia, and N_2O to N_2. NO is not utilized by whole cells, and no N_2 is formed from nitrate reduction (950). Other examples that fall into this category are the magnetotactic bacterium MV-1, which deposits magnetite crystals under N_2O respiration (46), and the nitrate-respiring but nondenitrifying *Campylobacter fetus* (644).

Reduction of N_2O has been reported for *E. coli* (434). The rates are reasonably high (30 nmol $\cdot$ min^{-1} $\cdot$ mg of protein^{-1}), and incorporation of ^{15}N from N_2O into N_2 was demonstrated. The reaction proceeds only at a very high N_2O concentration and is inhibited by nitrite and acetylene. The role of the process and the nature of the underlying enzyme are unclear.

During the development of a suitable assay system to detect life on Mars, it was found that acetylene inhibits the reduction of N_2O (239). The usefulness of acetylene for studies of denitrification was shown for pure cultures (35, 951) and became a popular and extremely useful technique to assay the denitrification potential of soil. Although inhibition by acetylene is not specific for N_2O reductase, acetylene is more selective than other inhibitors of denitrification (mostly metal chelators) and provides strong evidence for N_2O as an intermediate. The inhibition of N_2O reductase by C_2H_2 is noncompetitive, with a K_i of 28 to 45 μM (35, 472). Inhibition is reversible, but there is no experimental evidence to show where C_2H_2 binds and what its mechanism of action is. C_2H_2 also affects other metalloenzymes, including the putative Cu enzyme ammonia monooxygenase (reviewed in reference 376).

Isolation and Properties of N_2O Reductase

The search for N_2O reductase. For long time, N_2O reductase had been an elusive enzyme. After the acceptance of N_2O as an intermediate and formulation of the denitrification pathway, cell-free N_2O reduction still could not be demonstrated. Even to date, the assay for this enzyme is not developed to a degree that would allow the routine detection of the reductase in cell extract. The mere capability to grow a bacterium on N_2O (541) did not render a way for the isolation of the underlying enzyme. We had found a multicopper protein (which later became known as the pink form of N_2O reductase) as a byproduct of the purification of cytochrome cd_1 from *P. stutzeri* (982). The isolation of this Cu protein remained unreported since there was no enzymatic activity or known biological role, except for the spectral similarity of the *P. stutzeri* protein to a Cu protein of unknown function from *Alcaligenes faecalis* IAM 1015 (544, 547). Evidence for the role of the Cu protein as N_2O reductase came 3 years later from an entirely different direction.

Once Cu was recognized as a nutritional trace element for bacterial growth on N_2O, the key for seeking the reductase among bacterial Cu proteins was at hand. The requirement of Cu for N_2O respiration resides in the fact that N_2O reductase is a multicopper enzyme. In hindsight, the choice of the source organism was fortunate. Because *P. stutzeri* does not synthesize a cupredoxin, the Cu requirement could not be at the level of electron donation, and since this denitrifier has the cytochrome cd_1 nitrite reductase and not CuNIR, nitrite reduction is also independent of Cu. Media deficient in Cu will either not sustain cell growth on N_2O or at least lead to a transient accumulation of N_2O from nitrate (392, 394, 542). A Cu-containing protein of $\approx$120 kDa was found only in N_2O-utilizing *P. stutzeri* and *P. fluorescens* but not in *P. aureofaciens* and *P. chlororaphis*, which terminate constitutively denitrification at the level of N_2O (547). In an enzymatically coupled assay with hydrogen and clostridial hydrogenase for the reduction of BV, the Cu protein reduced N_2O to N_2, which marked the discovery of N_2O reductase as a molecular species (981). In addition to the biochemical evidence, Tn*5* mutants of *P. stutzeri* that failed to utilize N_2O did not synthesize the Cu protein and provided genetic proof of its essentiality in N_2O reduction (971).

Independent work with the enzyme from *Paracoccus denitrificans* gave a different molecular mass and showed no copper associated with N_2O-reducing activity; suggesting that a different type of reductase was present. The apparent separation of Cu from N_2O reductase, however, is an artifact of aerobic chromatographic conditions (970). Later, it was proven that the properties of the *Paracoccus* enzyme are not different from those of other N_2O reductases (68, 778).

Molecular properties. N_2O reductase has been purified and characterized to different extents from heterotrophic, phototrophic, and chemolithotrophic gram-negative bacteria, which usually have a soluble enzyme with a functional location in the periplasm. Table 8 compiles basic properties of these enzyme species. The most intensively studied enzyme is the one from *P. stutzeri*. N_2O reductases exist in several forms distinguished by their redox and spectroscopic properties, for which trivial names and a numerical classification are in use (Table 9). N_2O reductase isolated in the absence of oxygen (546, 970) is obtained as the purple species (form I) with an absorption maximum at 540 nm and a continuous-wave EPR spectrum at X band (9 GHz) with a seven-line hyperfine pattern in the $g_{\|}$ region (Fig. 20). The EPR spectrum is unusual for Cu proteins. It arises from an unpaired electron that is distributed over two equivalent Cu nuclei. Single isotope substitutions have proven that the hyperfine structure cannot be explained from the natural abundance of ^{63}Cu and ^{65}Cu in the enzyme and that nitrogen hyperfine splitting contributes negligibly to the EPR spectrum (604).

In the resting state, form I exhibits an intense absorption maximum around 540 nm ($\varepsilon \approx 16\ mM^{-1}\ cm^{-1}$), a shoulder at 480 nm, a low-intensity band at 350 nm, and a broad band at around 780 nm (Fig. 20). This absorption envelope is a composite of several Cu-S transitions originating from the mixed-valent Cu center that dominates the electronic spectrum (235). The positions of the absorption bands vary somewhat with the source of the enzyme and the method of preparation, but their overall shape makes them diagnostic for an N_2O reductase. Initially, N_2O reductase was purified in air. The enzyme thus obtained is the pink species, or form II, with both a lower activity and Cu content, presumably due to oxygen affecting the catalytic center. Form II has a low ratio of absorbances at 480 and 550 nm and a broad maximum at 630 to 650 nm not seen in form I. In a scattergram based on numerous preparations obtained over the years, high activity and high Cu content correlate with form I and not with form II. Form I is therefore considered to represent a native state of the enzyme.

The *Paracoccus denitrificans* N_2O reductase, as isolated, exhibits two broad confluent absorption maxima at 550 and 660 nm. Exposure to N_2O transforms the spectrum to that of the purple form (778). Further exposure to air or ferricyanide changes the enzyme into a form resembling the pink variant. N_2O reductase from *Rhodobacter capsulatus* is obtained as the pink form (553). The electronic spectrum of the reductase from *R. sphaeroides* differs from that of *R. capsulatus* and the other N_2O reductases. The far-red band with an extinction coefficient of $2{,}700\ M^{-1}\ cm^{-1}$ at 740 nm represents the strongest absorption relative to that at shorter wavelengths (572), whereas the absorption at 534 nm ($\varepsilon = 2{,}520\ M^{-1}\ cm^{-1}$) is substantially lower than that of other N_2O reductases. The enzyme is reported to contain two Zn and one Ni in addition to four Cu atoms per 75 kDa. The altered spectrum of this N_2O reductase may indicate that the heterometals occupy the Cu sites at least in part.

N_2O reductase is composed of two identical subunits. The subunit mass derived from the primary structure of several different sources is uniformly around 66 kDa. Monomeric enzyme forms have been suggested for the "*A. cycloclastes*" and *Rhodobacter sphaeroides* enzymes (Table 8) but might not hold up under closer scrutiny. The primary structures of N_2O reductases (NosZ proteins) show a tight clustering (Fig. 3) indicative of a homogeneous protein family with the same properties. The average Cu content of N_2O reductase is 4 Cu/65.8-kDa subunit, but because of the considerable uncertainty of chemical analysis for a high-molecular-mass protein containing several metal atoms, the exact number of Cu atoms in this protein may not be settled until the structure of the catalytic Cu center, currently assumed to be binuclear, is proven. An inactive enzyme obtained from the *P. stutzeri* MK402 mutant contains up to four Cu atoms per dimeric protein (form V). In this species, the Cu_A center is preferentially occupied, which was pivotal for elucidating the nature of this novel Cu site (219, 235).

The N_2O reductases from the nondenitrifiers *Rhodobacter capsulatus* and *W. succinogenes* have been investigated to see whether they would exhibit novel features compared to denitrifiers sensu stricto. The enzyme from the phototroph *R. capsulatus* has absorbance properties and subunit size similar to those of the Cu-containing N_2O reductase from denitrifying bacteria (553). In contrast, the *Wolinella* N_2O reductase exhibits the optical spectrum of a heme protein (Table 8). The EPR signal at $g_{\|} = 2.035$ with a six- to seven-line hyperfine splitting in the g region shows that a Cu_A center is present also (959). A signal at $g = 3.10$ can be attributed to ferric heme C; no signals of adventitious Fe or high-spin heme Fe are present. The enzyme activity is associated with an 88 kDa protein; this species is also observed in SDS-polyacrylamide gel electrophoresis and stains positively for heme. Chymotrypsin cleaves the *Wolinella* reductase into several fragments with lower masses, including a 13- to 14-kDa heme peptide representing the cytochrome domain of the enzyme (811). The enzyme is an interesting case with potential significance with respect to the evolution of Cu-heme centers. Its primary structure is not known. Identification of a heme-binding site of a gene-deduced single peptide is required to confirm the nature of the *Wolinella* enzyme as a Cu-heme protein.

The presence of heme may be responsible for the high specific activity of this N_2O reductase (811). The enzyme can use dithionite as an electron donor without a mediator and, other than the Cu-only N_2O reductases, is not immediately inactivated by this reductant. N_2O partially reoxidizes the heme of the dithionite-reduced enzyme. The reductase may have an intramolecular electron transfer chain with an electron entering at the heme and passing via the Cu_A center on to the catalytic site. Alternatively, the heme has been viewed as part of a heme-Cu center, with the three Cu atoms of the *Wolinella* reductase in seeming support of such a bimetallic center (811). However, in view of what is known of other N_2O reductases, a bimetallic site appears unlikely.

Electron donors. Electron transfer to N_2O reductase has been studied mostly with *Paracoccus denitrificans*, *Rhodobacter sphaeroides*, and *R. capsulatus*. The question of the electron transfer pathway toward N_2O reductase cannot be answered by naming a single distinct electron carrier. Parallel pathways of electron transfer and sometimes alternative electron donors for N_2O reductase exist in different denitrifiers. Absorbance changes on exposure of whole cells to N_2O indicated earlier a general involvement of *c*- and *b*-type cytochromes. The reductase does not interact directly with a respiratory complex but draws electrons from an intermediate carrier (540). In the case of the photodenitrifiers, the donor is cytochrome c_2 (387, 681), whose synthesis is increased under denitrifying conditions (573). Cytochrome c_2 is similar to mitochondrial cytochrome *c*; its redox potential is around +350 mV (929). In *R. capsulatus*, cytochrome c_2 is reduced by alternative routes, involving either the bc_1 complex or an ubiquinol oxidoreductase, bypassing cytochrome bc_1 (681).

Cytochrome c_{550}, which is present under all growth conditions, is the principal electron carrier from the bc_1 complex to sinks in the periplasm of *Paracoccus denitrificans*. The primary and secondary structures of this cytochrome are similar to

TABLE 8. Properties of N_2O reductases (EC 1.7.99.6)

Property	Value for:						
	Pseudomonas stutzeri	*Pseudomonas aeruginosa*	*Alcaligenes xylosoxidans*	*"Achromobacter cycloclastes"*	*Paracoccus denitrificans*	*Rhodobacter sphaeroides*	*Wolinella succinogenes*
Mol mass[a] (kDa)	120	120	120	72	143	95	162
No. and mass (kDa) of subunits	2, 65.8[b]	2, 65.9[b]		1, 72	2, 66.3[b]	1, 73–89	2, 88
pI	4.98, 5.04	5.2–5.7	6.2		4.3	4.9	8.6
Visible absorbance (nm)							
Protein as isolated	350, 480sh[c], 540, 780	480sh, 550, 630sh	360sh, 480sh, 545, 650sh, 780	350sh, 481, 534, 625, 780	350, 550, 550, 620sh, 820	481, 534, 635, 743	410, 528
Dithionite-reduced protein	650	670	650	650	660	640	416, 520, 550
ε (mM^{-1} cm^{-1}) [λ_{max}, nm]	14–16.3 [540]	11 [550]	6.5 [545]	5.3 [534]	13.3 [550]	2.5 [534]	150 [Soret][d]
No. of metal atoms/holoenzyme	8 Cu	8 Cu	5 Cu	4 Cu	8 Cu	4 Cu, 2 Zn, 1 Ni	6 Cu, 2 Fe
EPR (protein as isolated)							
$g_{\|}$	2.18	2.15	2.24		2.224	2.267	2.17
$A_{\|}$ (mT)	3.83 (7)[e]	4.5 (6)[e]	16 (2–3)[e]		3.5 (4)[e]	12.7 (4–5)[e]	4.5 (6)[e]
$g_{\perp}$	2.03	2.03	2.07	2.045	2.042	2.028	2.035
$A_{\perp}$ (mT)	2.8	2.5					2.6
EPR-silent Cu	+	+		+	+		+
Electron donor(s)	BV, MB[f]	BV		MV	BV, MV	BV	BV
Sp act (μmol of $N_2O \cdot min^{-1} \cdot mg^{-1}$)	60	27		86	122	25	160
K_m for N_2O (μM)		2			5–7	26	7.5
K_m for BV (μM)		4					4.0
Inhibitors	CN^-, N_3^-, NO, $S_2O_4^-$, C_2H_2	C_2H_2	CN^-, N_3^-, DNP, Cu^{2+}		CN^-, N_3^-, CO, C_2H_2	CN^-, N_3^-, EDTA, C_2H_2	CN^-, N_3^-, C_2H_2 (weak)
Activators	Base, CO	Base			BV		
Reference(s)	157, 684, 974	780, 781, 974	546	370	68, 349, 472, 778	572	811, 959

[a] By gel filtration.
[b] Sequence-derived value.
[c] sh, shoulder.
[d] Derived from Fig. 8 of reference 811.
[e] Number of lines resolved at X band.
[f] MB, methylene blue.

TABLE 9. Classification and properties of the different forms of N_2O reductase

Enzyme form	Trivial name	Molecular properties and observations
I	Purple	Prepared under exclusion of oxygen; ≈8 Cu/132 kDa; high catalytic activity; at low temperature the X-band EPR spectrum shows seven lines with $g_{\|}$ 2.18 and $g_{\perp}$ 2.03 and hyperfine coupling constants of $A_{\|}$ 3.83 mT and $A_{\perp}$ 2.8 mT, respectively; two intense, oppositely polarized transitions in MCD
II	Pink	Prepared in the presence of oxygen; lower catalytic activity than form I; altered electronic absorption spectrum from that of form I; EPR hyperfine structure less well resolved
III	Blue	Obtained under reducing conditions from forms I or II; broad featureless EPR signals at 9.32 and 34 GHz; resonance Raman feature of type 1 Cu; opposite polarized MCD bands red shifted
IV	Reconstituted	Prepared from apoenzyme by incubation with Cu(II); catalytically inactive; EPR properties similar to those of form V; electronic absorption properties of a Cu_A protein
V	Mutant	≈4 Cu atoms/132 kDa; obtained from *P. stutzeri* MK402 with the operon encoding the Cu insertion system inactivated; a Cu_A protein with spectroscopic features similar to the Cu_A peptides of *B. subtilis* and *P. denitrificans* oxidases, or the engineered Cu_A cupredoxins of amicyanin or azurin

those of cytochrome c_2 and the mitochondrial cytochrome *c*. Inactivation of the gene for cytochrome c_{550} does not affect N_2O reduction or nitrite reduction, because periplasmic carriers can substitute each other (872). An alternative carrier is proposed to be a blue type 1 Cu protein, which makes this situation analogous to that for cytochrome cd_1. Purified N_2O reductase from *P. denitrificans* GB17 accepts electrons from pseudoazurin and cytochrome c_{551} and also from horse heart cytochrome *c* in vitro (68). The mutant C010 of *P. denitrificans* PD1222, which synthesizes no cytochrome c_{550}, is not affected in N_2O reduction, but the reaction becomes partially sensitive to DDC. This suggests electron transfer to N_2O reductase via a Cu protein, presumably the pseudoazurin of this organism (587). Note that there is no need for a low-potential donor to N_2O reductase in accordance with an earlier observation that methylene blue serves as artificial electron donor (157). The reactivity of the same reductase with different types of electron carriers manifests a low degree of recognition specificity at the electron entry site.

A monoheme *c*-type cytochrome (9.2 kDa) isolated from *W. succinogenes* is active with the reductase from this bacterium and reduces N_2O with second-order kinetics (958). Evidently there is no tight complex formation between the electron donor and the enzyme; the electron transfer from the cytochrome rather than substrate reduction is the likely rate-limiting step. Cytochrome c_{551} from *P. aeruginosa* [E_0'(pH 7) = +286 mV (361)] is also active with *Wolinella* N_2O reductase although to a lesser degree.

Copper Centers

The Cu sites of N_2O reductase have been proposed as two binuclear centers in each one of the two enzyme subunits. They are the EPR-detectable, mixed-valent [$Cu^{1.5}$...$Cu^{1.5}$], S = 1/2, Cu_A site and the EPR-silent, antiferromagnetically coupled [Cu(II)...Cu(II)] Cu_Z site (236). Whereas ample structural evidence exists for the binuclear nature of the Cu_A site, the elucidation of the Cu_Z site is still in progress. The model of two binuclear sites accommodates the molecular properties of native N_2O reductase and that of the mutant form V, the analytical data with respect to the amount of Cu and the EPR-detectable Cu in the various forms and redox states of the enzyme, as well as a wealth of spectroscopic evidence obtained from multifrequency EPR, electron spin-echo envelope modulation, UV-visible spectroscopy, circular dichroism, MCD, EXAFS, and resonance Raman spectroscopy.

Cu_A, a novel type of metal center. Cu_A has unique properties among the Cu centers of proteins and was firmly believed to be exclusive for COX with a mononuclear $Cu(Cys)_2(His)_2$ structure (132, 274, 513, 537). The study of Cu_A provides an illustrative case that progress in science sometimes comes unexpectedly from a seemingly unrelated area. The term "Cu_A" was applied to N_2O reductase on recognizing the spectroscopic and structural similarity with COX (475, 737). There is compelling evidence now that N_2O reductase and COX share in Cu_A a structurally homologous metal center. The unraveling of the binuclearity of Cu_A of N_2O reductase by spectroscopic techniques was decisive for recognizing the same structure in subunit II of COX.

The seminal finding for a relationship between N_2O reductase and COX was the identification of a set of potential Cu ligands in the C-terminal domain of N_2O reductase that correspond to those of the Cu_A-carrying subunit II of COX (878). Without exception, the same ligands are present in the deduced primary structures of other N_2O reductases (Fig. 21). Various spectroscopic techniques subsequently reinforced this relation (236, 408, 737). EXAFS gave several Cu-N and Cu-S distances for N_2O reductase that were already known from COX, including an unusually large Cu-S distance in both enzymes (737). This distance was recently confirmed with the soluble Cu_A fragment from *Bacillus* COX, but was reinterpreted as a 2.5-Å Cu-Cu metal bond (78). Although the very first EXAFS data from N_2O reductase did not exclude such a possibility (737), a Cu-Cu interaction is nearly indistinguishable from Cu-S scattering, and EXAFS alone provides no clear distinction of a bridged from a terminal configuration of coordinating thiolates in N_2O reductase (139).

The interpretation of the Cu signal in the EPR spectrum of COX dates back to 1962. Its history was put into the perspective of the analytical data for Cu and functional concepts at that time, which led to the rejection of a Cu-Cu site (53). A binuclear Cu *bis*-acetato complex (764), not COX, served as the model to explain the seven-line EPR spectrum of N_2O reductase within the framework of mixed valency (475). The hyperfine structure is resolved, with N_2O reductase at both low and high field in the EPR spectrum because there is no overlapping heme signal as in the oxidase. Low-frequency EPR, particularly C band (4.5 GHz), achieved a previously unknown resolution of Cu signals in the high-field region of COX. On realizing that the *g* values can be calculated consistently for various frequencies only by assuming a binuclear Cu site, Kro-

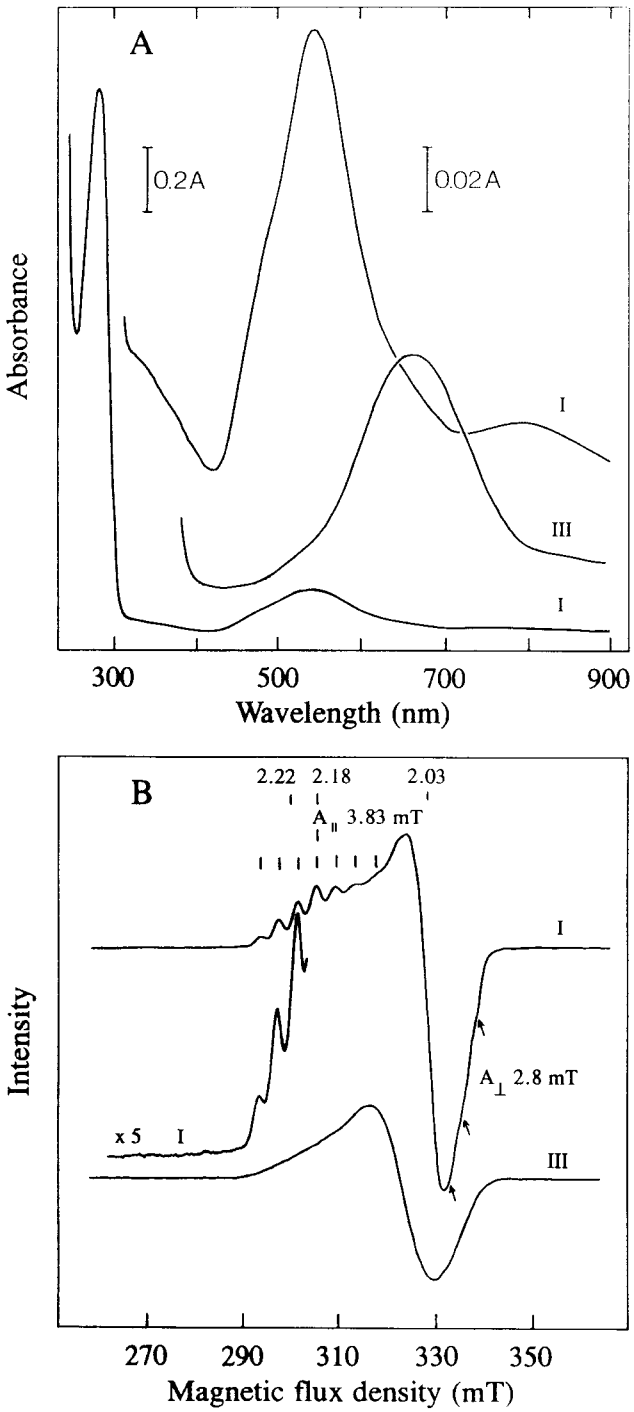

FIG. 20. Electronic absorption (A) and X-band EPR (B) spectra of the two principal forms of N_2O reductase from *P. stutzeri* ZoBell. I, Resting or purple form; III, blue form obtained by the addition of dithionite to form I. Conditions of recording for EPR spectra: microwave frequency, 9.31 GHz; modulation frequency, 100 kHz; modulation amplitude, 0.5 mT; scan rate, 0.2 mT s^{-1}; microwave power, 1.6 mW; temperature, 10 K. Reproduced with permission from references 970 and 157.

neck et al. (476) and Antholine et al. (16) concluded that the Cu coordination found in N_2O reductase is applicable to COX and that the Cu_A center in both enzymes is a mixed-valent site of two interacting Cu nuclei. Their spectroscopic data were deemed to have established beyond doubt the binuclear nature of the Cu_A center (74). Parallel to the spectroscopic work, it was shown that COX has a stoichiometry of three Cu atoms per two heme groups (787), removing the impediment that had led to the rejection of a binuclear site at the very beginning of this problem (53, 55). The unfolding of the Cu_A story since its beginning was related recently by H. Beinert, a pioneer in the field (54).

The candidate ligands of the Cu_A center were identified from site-directed mutagenesis of Cu_A fragments of terminal oxidases (234, 445) and N_2O reductase (973). They correspond to the two cysteines, two histidines, and one methionine predicted as ligands from comparative sequence analysis of N_2O reductase and subunit II of COX (974). The C-terminal domains of NosZ proteins exhibit a conserved set of ligands and identical spacing to that of subunit II proteins, covering sources from bacteria to humans (Fig. 21). The structural conservation of the Cu_A domain in the two types of proteins is probably pronounced in the secondary structure. A number of residues strictly conserved within the subunit II are not found in N_2O reductase. Beyond the Cu_A domain N_2O reductase and COX do not show sequence relatedness.

Substitution of the Cu_A-binding amino acids and of Asp580 affects N_2O reductase activity, as expected for an essential role in metal coordination. Aspartate has been suggested to provide an oxygen ligand to the Cu_A center (604), but this is still hypothetical. The homologous residue of *P. denitrificans* COX, Asp178 (Asp206 in the unprocessed sequence), is hydrogen-bridged to the Cu-coordinating His181 (395). Asp178 was suggested to be part of the cytochrome *c*-binding site (504, 914). Mutagenesis data for N_2O reductase, which show activity modulation depending on the nature of substitution, are more compatible with this latter function than with Asp580 being a ligand to Cu (225).

Figure 22 shows a model for the Cu center of N_2O reductase based on EPR data and ligand assignments from site-directed mutagenesis. The atomic distances are based on the EXAFS properties of form V of the reductase (139). Whether or not the Cu pair in the Cu_A center is bridged by cysteines remained unsolved until very recently. Both variants of bridging and terminal thiolates were considered for N_2O reductase and COX, since the mode of cysteine coordination was not clearly differentiated by spectroscopic means of probing the Cu_A center. The determination of the crystal structures of bacterial and bovine COXs (395, 839) and the engineered Cu_A fragment from the bacterial CyoA quinol oxidase (908) resolved this question in favor of a doubly cysteine-bridged structure and confirmed the binuclearity of the Cu_A center at the same time. Without the necessity of invoking a metal-to-metal bond (74), the Cu_A structure in Fig. 22 accounts for the high electron delocalization as seen in EPR (16) and the close Cu-Cu distance from EXAFS (78, 139).

The knowledge gained from the Cu_A structure of the oxidases can be applied tentatively to the structural model of Cu_A for N_2O reductase until a crystal structure becomes available for the latter. The two Cu atoms and the two cysteine sulfur atoms form a nearly planar Cu_2S_2 rhomb in the oxidase. The Cu-S-Cu angle is $\approx$70°. The ligands around each Cu form two distorted tetrahedrons, which share an edge along the S-S direction. The nitrogen atoms from the two coordinating histidines are approximately in a linear arrangement and have a common plane that may be slightly tilted with respect to the Cu-Cu axis. In the CyoA fragment, this angle is 15° (908). The crystal structure revealed unexpectedly a carbonyl group from the main peptide chain as a ligand to the Cu_A center. The equivalent position in N_2O reductase is occupied by trypto-

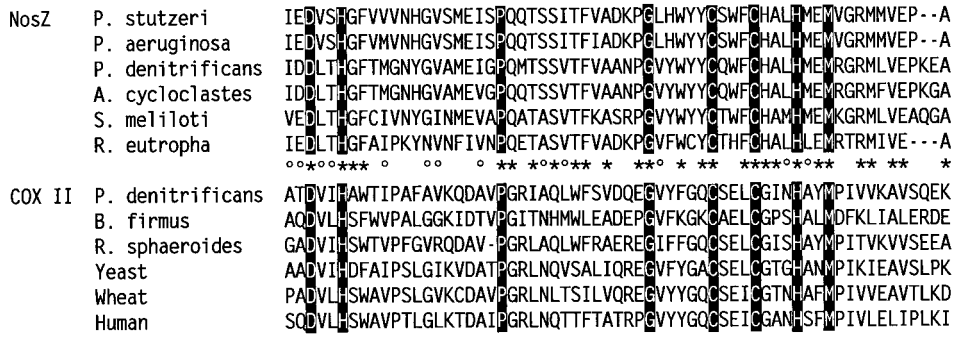

FIG. 21. Alignment of the Cu_A-binding domain of bacterial N_2O reductases (NosZ) and COX II. Residues that are identical among all sequences are shown in negative print. The extent of sequence conservation among the NosZ sequences is shown by ∗ and ○, denoting identical and conserved residues, respectively. The C-terminal amino acid of all NosZ sequences is alanine; the sequences of COX II are C-terminally incomplete. Sequence sources, SwissProt data bank; "*A. cycloclastes*" (556).

phan and in one instance (*R. eutropha*) by a histidine. The oxo group of the backbone carbonyl in the CyoA fragment is at a 2.28-Å distance; the EXAFS fits indicate a distance of 2.05 Å, for N_2O reductase, whereas in the *Paracoccus* structure the oxygen atom is at ≈3 Å. Although the main features are identical in the different Cu_A centers, there are variations and subtle structural differences (235, 603). A model of the Cu_A site with a $[Cu_2(\mu\text{-}SR)_2]^+$ core was recently synthesized (365). The complex has the geometry of a resting Cu_A site in proteins and exhibits its strong electron delocalization.

The Cu_A domain exerts a strong influence on the structural integrity of N_2O reductase. Deletion of the domain renders N_2O reductase unstable and subjects it to rapid degradation. Certain mutations of Cu_A ligands affect protein export and result to various degrees in the accumulation of a cytoplasmic N_2O reductase (973). This indicates that the folding of N_2O reductase into its transport-competent form is affected by the Cu_A domain. Similar observations have been made for mutations in the Cu_A domain of yeast COX (782). Substitution by other potential metal ligands or impeding of Cu binding by a small perturbation of the site reduces the amount of immunodetectable subunit II. Since translation continues normally, misfolding and degradation of the subunit are consequences of certain modifications of the Cu_A center.

Engineered Cu_A proteins. N_2O reductase and COX are to date the only two naturally occurring proteins for which a Cu_A site has been identified. An important development was the introduction of the ligands for Cu_A into a water-soluble fragment of the CyoA quinol oxidase, which generated a binuclear purple Cu center (864) and provided the source of the first high-resolution crystal structure (908). The electron density map resolves both Cu atoms at a 2.48-Å distance, which corresponds to the EXAFS measurements (78, 139, 331). Water-soluble Cu_A domains, truncated from the membrane-spanning part, of *Paracoccus denitrificans* COX (503), the *caa*$_3$-type oxidase from *B. subtilis* (884), and cytochrome *ba*$_3$ from *Thermus thermophilus* (773) have been engineered and heterologously expressed in *E. coli.* In all cases, an apoprotein was obtained in which a purple Cu_A site was generated by incubation with Cu. The UV-visible absorption properties of the reconstituted proteins are similar to those reported for N_2O reductase after cloning and expression of *P. stutzeri nosZ* in *E. coli* and insertion of Cu (Fig. 23) (878). EPR suggests a mixed-valent nature for the soluble Cu_A domains even though they were reconstituted with Cu(II) (240, 503, 884). In most cases the EPR hyperfine structure of the Cu_A proteins, including form V of N_2O reductase, is less well resolved than for N_2O reductase form I. However, the S-band (2.5 GHz) EPR spectra of form I and form V are nearly identical, showing the same paramagnetic species, i.e., Cu_A, (Fig. 24). Analogs of the Cu_A site have been engineered by extending the cupredoxin scaffolds of amicyanin (199) and azurin (325) with a peptide loop carrying the Cu_A ligands. It was previously noted that the Cu_A domain of N_2O reductase has structural similarity to blue type 1 Cu proteins (974). The possibility to engineer a Cu_A site into cupredoxins lends support to expectation of a β-sheet fold for Cu_A in N_2O reductase.

There are many questions about the Cu_A center to which currently only fragmentary answers are possible. What is the rationale behind binuclear electron transfer centers in N_2O reductase and COX? Will Cu_A also be found in other proteins? Is the Cu_A site required for a particular pathway and/or to bridge the distance in intramolecular electron transfer? What are the functional aspects in the electron-transferring properties of a binuclear versus a mononuclear Cu center, and what are the distinct electronic properties of Cu_A? An evolutionary link has been proposed for Cu_A-containing enzymes which may explain its extant distribution in the two types of oxidoreductases (714, 863). A once wider occurrence may be deduced from the hypothesis that a Cu_A center had been part of the quinol oxidases (864). Engineering a type 1 site into Cu_A proteins is an intriguing approach to gaining insight into the ne-

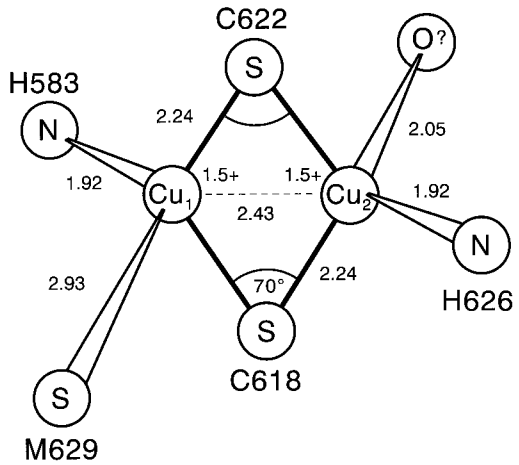

FIG. 22. Model of the Cu_A center of N_2O reductase. Ligands are assigned from site-directed mutagenesis; the oxygen ligand to $Cu_{(2)}$ is suggested from EXAFS but is so far not attributed to a specific residue by mutation. The distances are derived from EXAFS on form V of N_2O reductase from mutant MK402 (Table 9).

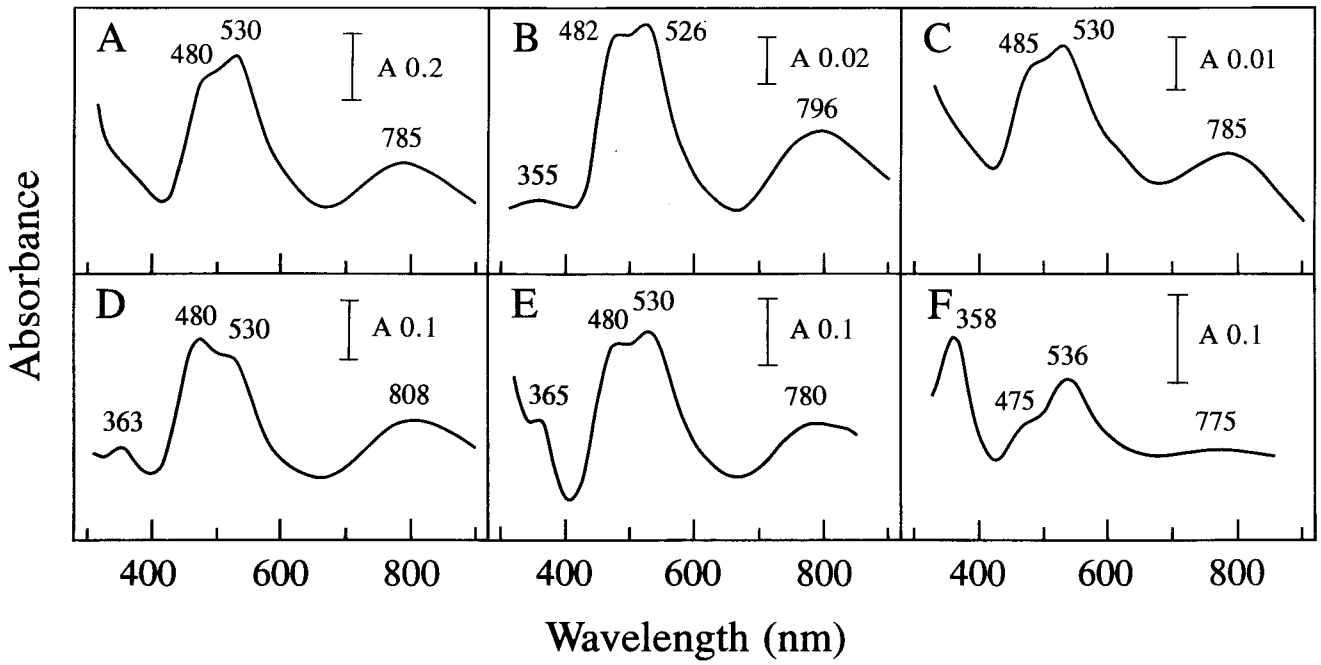

FIG. 23. Electronic absorption spectra of Cu_A proteins. (A) Apo-N_2O reductase from *P. stutzeri* reconstituted with $Cu(en)_2SO_4$ (157); (B) N_2O reductase as isolated from *P. stutzeri* MK402 defective in Cu chromophore assembly (684); (C) heterologous N_2O reductase from *E. coli* HB101(pAV5018) reconstituted with $Cu(en)_2SO_4$ (878); (D) engineered soluble Cu_A-binding domain of COX of *Paracoccus denitrificans* reconstituted with $CuCl_2$ (503); (E) engineered soluble Cu_A-binding domain of COX of *B. subtilis* reconstituted with $CuCl_2$ (884); (F) water-soluble, engineered peptide from cytochrome *o* quinol oxidase of *E. coli* carrying a $CuCl_2$-reconstituted Cu_A site which was introduced by site-directed mutagenesis (864). Reproduced with permission from the indicated references.

cessity for the binuclear site (962). A certainly remarkable property of Cu_A in N_2O reductase is its extremely short relaxation time (645). The Cu_A rhomb Cu_2S_2 has almost aromatic properties with a complete electron delocalization (603). Unlike aromatic systems, with delocalization by the out-of-plane π system, the delocalization here is via the in-plane σ system.

Most intriguing is the view of the Cu_A center as the inorganic analog of a quinone with the mixed-valent state representing the semiquinone form (235). Although redox measurements indicate a one-electron donation from Cu_A, a two-electron donation may be possible. Spectrophotometric titration of N_2O reductase at 540 nm with ferricyanide as the mediator gives a midpoint potential, E_0', of +260 mV (pH 7.5 and 25°C) with a Nernst factor of 0.95 (157). The potential corresponds to that of Cu_A from COX (531). A reversible oxidation of the mixed-valent species to the fully oxidized form, [Cu(I). . .Cu(II)] $\rightleftharpoons$ [Cu(II). . .Cu(II)], preserving the integrity of the center, has not been achieved.

Cu_Z, the catalytic site. N_2O reductase catalyzes the two-electron reduction of N_2O to N_2 and water: $N_2O + 2H^+ + 2e^- \rightarrow N_2 + H_2O$ [E_0'(pH 7.0) = +1.35 V; $\Delta G^{\circ\prime} = -339.5$ kJ/mol]. Thermodynamically, N_2O is a potent oxygen transfer reagent (358), yet in the absence of an activating agent (usually a transition metal) it is extremely inert, comparable to N_2 (37, 422). N_2O is also a poor ligand (97). With few exceptions, N_2O tends to react with transition metal complexes by extrusion of N_2 and formation of oxo complexes $M^n + N_2O \rightarrow O{=}M^{n+2} + N_2$ (see references 478 and 874 and citations therein). $[Ru(N_2O)(NH_3)_5^{2+}]$ remains to date the only stable N_2O transition metal complex where N_2 is not immediately extruded (27, 28). The crystal structure of this complex is not known, but circumstantial evidence favors a terminal N-bonded structure (840). N_2O can be bound in N_2O reductase end-on to a Cu atom or as a bidentate ligand of a binuclear site. Unidentate binding to Cu may occur via nitrogen or oxygen. A considerable amount of electron density in the resonance structures of N_2O at either the oxygen or the nitrogen ($N{\equiv}\overset{+}{N}{-}\bar{O} \leftrightarrow \bar{N}{=}\overset{+}{N}{=}O$) makes both ways of binding feasible. Bidentate coordination oriented by the surrounding protein environment to a binuclear Cu_Z site could be a means of weakening the N—O bond and achieving oxygen transfer to one Cu atom and release of dinitrogen from the other.

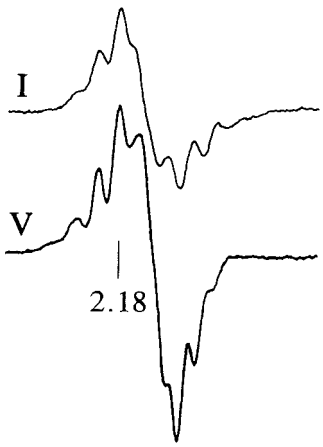

FIG. 24. S-band EPR spectra of forms I and V of N_2O reductase from *P. stutzeri* ZoBell. Conditions of recording for form I: microwave frequency, 2.393 GHz; microwave power, 20 mW; time constant, 0.3 s; scan time, 3 s/mT. Conditions for form V: microwave frequency, 2.792 GHz; microwave power, 0.26 mW; time constant, 0.03 s; scan time, 15 s/mT. Other conditions: modulation frequency, 100 kHz; modulation amplitude, 0.5 mT; temperature, 15 K. Both proteins were in 50 mM Tris-HCl (pH 7.5). Courtesy of P. M. H. Kroneck.

Reactivity of N_2O with metalloenzymes other than N_2O reductase, which contain Cu, Fe, Co, Ni, or Mo, has been observed (226, 231, 403, 522). N_2 is the product of N_2O in the nitrogenase, CO dehydrogenase, and methionine synthase reaction. The first two enzymes act as N_2O reductases, whereas the turnover of methionine synthetase under N_2O is inhibited because of $OH^{\bullet}$ radical formation (226). The enzymes that transform N_2O, in what must be considered nonphysiological reactions, provide no insights into the binding of N_2O to any of the metal centers involved but clearly show that in the appropriate redox environment activation of N_2O is not that exceptional and is possible with different transition metals.

If oxygen atom transfer takes place during the catalytic cycle of N_2O reductase, one expects from chemical precedence that N_2O reductase would be a molybdoenzyme. More enzymatic and nonbiological systems are known for oxo transfer to molybdenum than for any other transition element. Cu is notably absent from metal-oxo chemistry because this involves, as a rule, no more than 4d electrons and M═O groups being stabilized at metal centers with an oxidation state above +4 (358). However, as pointed out above, nitrite is an example of an N oxide species that appears to be activated by binding via the oxygen to the type 2 Cu of nitrite reductase.

The current concept of two binuclear sites in N_2O reductase considers Cu_A to be the site of electron entry from its physiological donor and Cu_Z to be the substrate (N_2O)-binding center. The K_m for N_2O is in the low micromolar range (Table 8). Compared with Cu_A, the structural information about Cu_Z is scant. Interaction of N_2O with Cu_Z has not been demonstrated. The *Paracoccus denitrificans* enzyme as isolated is in a partially reduced state and is oxidized by N_2O. This is the only reported instance where interaction with the substrate leads to a spectral change (778). The reactions with the inhibitor acetylene and activator CO do not lead to spectral changes of the enzyme, but binding of the inhibitors NO and CN^- and the substrate analogs N_3^-, NCO^-, and CNS^- can be monitored spectroscopically (258, 684). Exogenous ligands may provide tools to differentiate the Cu centers in N_2O reductase. Azide appears to bind to Cu_A and a modified catalytic site, but not at the intact, oxidized Cu_Z center; NO binds preferentially both to oxidized Cu_Z and to its semireduced state, but less to Cu_A (217). A second EPR signal seen at Q-band frequency (35 GHz) in form I but not in form V (16, 684) is tentatively attributed to a modified catalytic center (Cu_Z^* in the nomenclature of reference 236). Purple N_2O reductase also reacts with excess hydrogen peroxide or superoxide to form a blue transient of unknown structure (684).

N_2O reductase shows thermochromic behavior. On freezing, the purple and pink forms are slowly converted to a blue form. At high pH, the process is accelerated, and it is also more pronounced for the *Alcaligenes* than for the *Pseudomonas* enzyme (157, 546, 981). When the blue form of *P. stutzeri* is heated at 40°C for several hours, the pink form of the enzyme is restored. The difference spectrum of the heated minus the blue form corresponds to that of a pure Cu_A protein (unpublished data). The reversible changes in absorption properties imply structural alterations and/or oxidation-reduction processes of Cu_A and may be useful to reveal features of Cu_Z.

In the resting state of the reductase, only about 25% of the Cu are EPR active (157, 218, 684) because of the antiferromagnetically coupled Cu_Z center and the mixed-valent nature of Cu_A. The formalism of the two-center, binuclear model requires the addition of two electrons to the N_2O reductase dimer to generate the semireduced enzyme form. This form is EPR silent but displays low-energy, intense electronic transitions at 540 and 650 nm, which are attributed to Cu_Z and increase the 540-nm absorption peak of form I of the reductase with respect to the pure Cu_A spectrum (217, 236). On further addition of two electrons per dimer, Cu_Z is converted into an S = 1/2 EPR-active [Cu(I). . .Cu(II)] center (236). Experimentally, twice the number of electrons is taken up by N_2O reductase to produce these states (684). To resolve this discrepancy, sulfur redox chemistry at the active site has been postulated (477). Disulfide bridges critically placed near the Cu sites could account for the excess of electrons taken up on reductive titration. Reaction of N_2O reductase with the sulfhydryl reagent 5,5′-dithiobis(2-nitrobenzoic acid) yields 18 or 19 −SH groups/dimer, in agreement with the 18 cysteines derived from the *nosZ* gene (224). These residues are buried, and the reaction proceeds only after unfolding of the enzyme with guanidinium chloride. Holo-N_2O reductase has four disulfide bridges, which are likely to be introduced into the protein in the periplasm by a protein disulfide isomerase.

N_2O reductase is reducible photochemically with deazaflavin and oxalate or by borohydride, dithionite, thiols (e.g., glutathione), Fe(II)-$EDTA^{2-}$, and Cr(II) complexes (684). Irrespective of the nature of the reductant, the chromophore of purple N_2O reductase is first bleached rapidly, and in a slower process the blue species with an absorption maximum around 650 nm ($\varepsilon \approx 8\ mM^{-1}\ cm^{-1}$) appears (form III, Fig. 20). The blue species is redox inert, and no further reduction takes place even with excess reductant. Form III is also relatively stable toward autoxidation compared with the multicopper enzyme ascorbate oxidase, which has a trinuclear type among its Cu centers. The slow formation of form III casts doubts on its catalytic competence. The blue species is catalytically inactive and requires reoxidation to regain its activity (157).

Larsson et al. (505) have argued that the reorganization energy is lower for a binuclear than for a mononuclear site during the redox process and that this allows the fast electron transfer from Cu_A to heme A in COX. Since their argument is equally valid for N_2O reductase, the slow appearance of form III on reduction of N_2O reductase indicates that the electron transfer from Cu_A to Cu_Z is inhibited in the resting enzyme. Possibly this is relieved by a change in enzyme conformation and/or ligand binding. N_2O reductase is activated at high pH (68, 157, 780), which has been interpreted as a change in protein conformation (408) and may affect the electron transfer rate between the two Cu centers. During turnover, N_2O reductase is inactivated with a $t_{1/2}$ of ≈5 min (157, 778, 811). Whether this involves the formation of form III or another mechanism of blocking the active site is unknown.

Resonance Raman, circular dichroism, and MCD data indicate that there is a highly covalent thiolate Cu-S site in form III (10, 219, 220, 236). The MCD spectrum, with two intense oppositely polarized transitions, suggests a similarly binuclear and S-bridged structure to that for Cu_A. The nature and origin of this sulfur are not clear. There is one single conserved cysteine outside the Cu_A domain in three N_2O reductases. Its mutation destabilizes but does not inactivate the enzyme; hence, this residue cannot be part of the Cu_Z center (224). In the sequence from "*A. cycloclastes*," the homologous cysteine residue is lacking or shifted to a different position (556). The situation with the Cu_Z center of N_2O reductase resembles cytochrome cd_1 insofar as there is no common set of conserved metal center residues among proteins from distinct sources.

Eight histidines at positions 129, 130, 132, 178, 326, 382, 433, and 494 (*P. stutzeri* count) are conserved in the known four primary structures. This favors the contribution of multiple histidines to the coordination of Cu_Z. The substitution of His494 results in an inactive enzyme (unpublished data), and

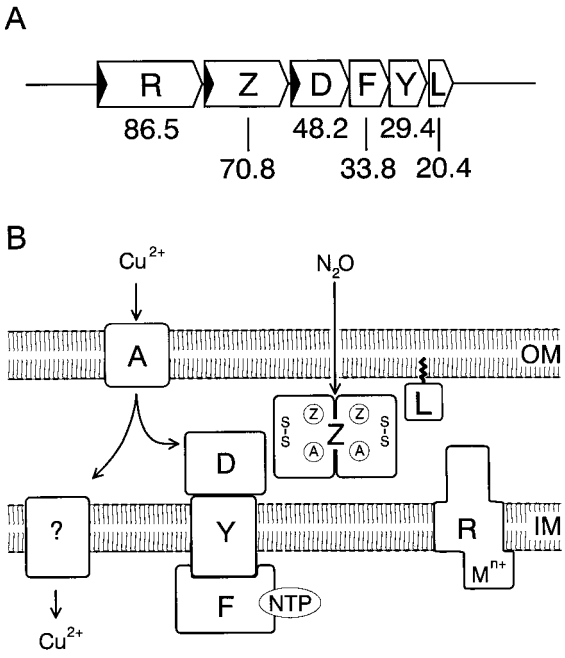

FIG. 25. Topology of the apparatus for copper insertion into apo-N_2O reductase. (A) Genetic organization of the *nosRZDFYL* genes with the promoters indicated by solid triangles. The molecular masses of the derived gene products are given in kilodaltons. (B) A, D, F, L, R, Y, and Z denote proteins NosA, NosD, NosF, NosL, NosR, NosY, and NosZ, respectively. The encircled small capitals A and Z represent the two types of Cu centers, Cu_A and Cu_Z. The question mark symbolizes a presumed copper transporter. OM and IM, outer and inner membranes, respectively. NTP, nucleotide-binding site of NosF; M^{n+}, putative metal-binding site of NosR. The model combines data from *P. stutzeri* ZoBell and JM300 (171, 172, 509, 588, 984). For discussions of the individual components, see the text. Reproduced with permission from reference 980.

further site-directed mutagenesis ought to address the other candidate ligands.

Assembly of the Copper Centers

The mechanism of insertion of a prosthetic metal into the cognate apoprotein has become an amenable problem due to the power of genetic manipulation of DNA regions that encode the necessary functions. Several cases where the metal is inserted in a catalyzed process, such as Fe into iron-sulfur proteins, Mo into nitrogenase, or Ni into hydrogenase and urease, have been studied (reviewed in reference 324). For N_2O reductase, the requirement of ancillary proteins catalyzing metal insertion was deduced early from genetic evidence (984). For an exported metalloprotein, the compartment of metal insertion has to be identified, as do the factors that confine the biosynthesis of the metal center to a particular site. Important elements in this process are the nature and availability of a metal donor, the site of folding of the metal center, and ancillary components for folding and metal insertion. Insertion of Cu in vitro into apo-N_2O reductase yields a protein with only a reconstituted Cu_A center, whereas no conditions have been found that would allow the reconstitution of the Cu_Z center (157, 684, 980). Occupancy of the Cu_A center in the mutant form V is best when cells are grown with 10 instead of 1 μM Cu; although this does not argue against a catalyzed process in vivo, it shows that spontaneous Cu incorporation is possible and that the problem of inserting Cu into N_2O reductase rests mainly with the Cu_Z center.

The ability to grow *P. stutzeri* on N_2O allowed the screening of random mutations and identified a group of mutants that synthesized a nonfunctional N_2O reductase (971, 984). The targeted DNA region is located downstream next to *nosZ* and comprises the genes *nosD*, *nosF*, and *nosY*. Their products encode a putative Cu insertion complex with components in and on either side of the membrane (Fig. 25). Preliminary topological information from *phoA* and *lacZ* fusions places NosF in the cytoplasm and NosD in the periplasm (171, 225).

The *nosY* product is highly hydrophobic and represents an integral membrane protein with six predicted transmembrane helices and no significant hydrophilic domains (Fig. 26). An α-helical content of over 50% is indicated from tertiary-structure analysis. NosY is similar in its topology to the homodimeric or heterodimeric membrane subunits of the family of ABC transporters, where each has six transmembrane segments. The degree of oligomerization of NosY, if any, is not known. The linkage of *nosD* with *nosZ* in *P. aeruginosa*, *Paracoccus denitrificans*, *S. meliloti*, and "*A. cycloclastes*" indicates the general existence of insertion complexes in N_2O-utilizing denitrifiers (349, 357, 556, 974).

Copper is thought to pass through the outer membrane via a pore formed by the NosA protein (508, 509). The mutational absence of NosA in *P. stutzeri* JM300 is associated with the formation of enzymatically inactive N_2O reductase that lacks copper (588). A homolog of NosA is OprC of *P. aeruginosa*, with 65% sequence identity (945). The *nosA* gene maps at a separate locus not linked to any denitrification gene clusters (Fig. 5). The NosA/OprC proteins form voltage-gated channels with a slight preference for Cu. The binding of one to three Cu atoms by these proteins has been reported. The primary structure does not reveal sequence motifs indicative of where the Cu may be bound. NosA and OprC are synthesized in a precursor form for export; the mature forms are 70.2 and 73.4 kDa, respectively. Their expression depends on nitrate and anaerobic conditions. An FNR box is located in the promoter region of *oprC* that points to the transcription factor ANR as anaerobic regulator (945). The synthesis of both proteins is repressed if the Cu concentration in the medium is above 10 μM. Repression of *oprC* is more sensitive to Cu than is that of *nosA*; in turn, *nosA* is repressed by Cu more strongly in cells grown under N_2O than in those grown under nitrate.

NosA has a high content of hydrophobic amino acids that may form β-sheets traversing the outer membrane in form of a pore. NosA is also a receptor for bacteriophages, and loss of phage sensitivity has been used as a convenient method to isolate mutants defective in N_2O respiration (150). Certain

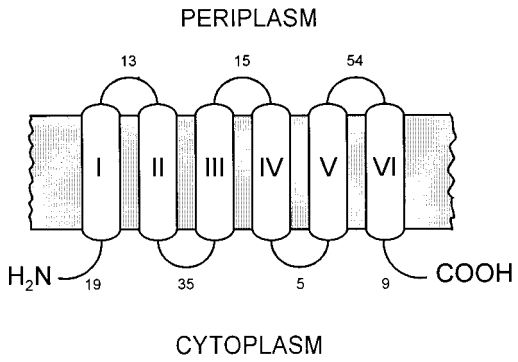

FIG. 26. Model of the topology of NosY from *P. stutzeri*. Six transmembrane helices, I through VI, are predicted; arabic numerals give the number of amino acid residues forming the periplasmic and cytoplasmic loops between the helices. The topology is derived from the TopPredII algorithm (151).

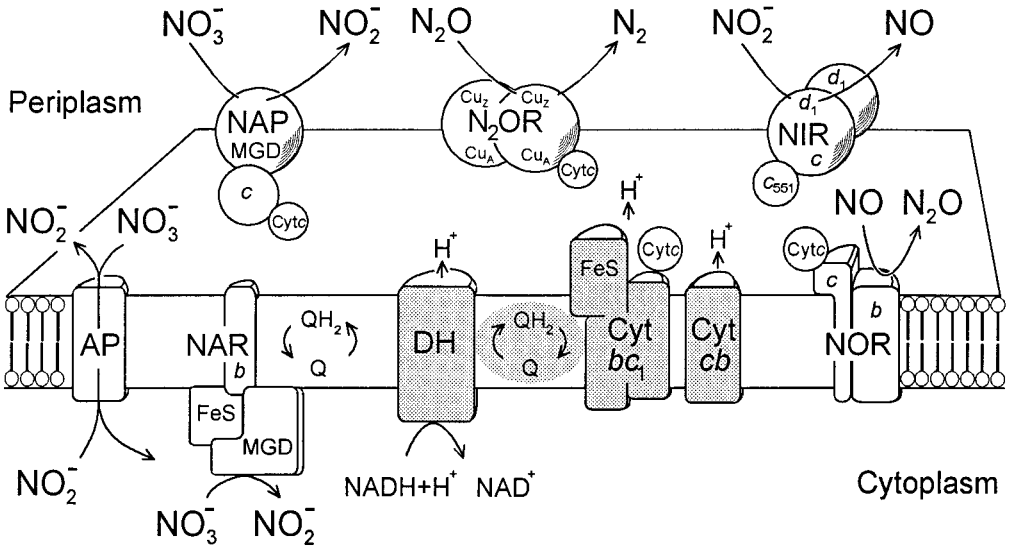

FIG. 27. Organization and sidedness of the anaerobic electron transfer chain of *P. stutzeri*. The shaded areas represent the components of the constitutive aerobic respiratory chain consisting of an NADH dehydrogenase complex (DH), quinone cycle (Q, QH_2), cytochrome bc_1 complex (Cyt bc_1), and the cytochrome *cb* terminal oxidase complex (Cyt *cb*). The denitrification system comprises respiratory nitrate reductase (NAR), nitrite reductase (NIR), NO reductase (NOR), and N_2O reductase (N_2OR). Evidence for the existence of the periplasmic nitrate reductases (NAP) in *P. stutzeri* is derived from DNA hybridization; the enzyme is modeled according to the situation with *R. eutropha* and *Paracoccus denitrificans*. Abbreviations: FeS, iron-sulfur centers; *b*, *c*, and d_1, heme B, heme C, and heme D_1, respectively; cyt *c*, unspecified *c*-type cytochromes accepting electrons from the bc_1 complex and acting on N_2OR and NOR; cyt c_{551}, cytochrome c_{551}; AP, postulated nitrate/nitrite antiporter.

nosA mutations are remedied by exogenous Cu, which hints at a further component acting in concert with NosA. Limited sequence similarity is observed between NosA/OprC and TonB-dependent outer membrane receptors for siderophores or vitamin B_{12}. NosA is hypothesized to be part of a Cu ion or Cu chelate uptake system (508). The repression of NosA/OprC by very low Cu concentrations is indicative of these proteins being part of an uptake system. Only a few examples of Cu-binding siderophores and a Cu-binding protein are known to be extracellular chelators of Cu (980). The growth requirement of *P. stutzeri* for Cu is satisfied in a synthetic medium by a concentration as low as 1 μM and suggests the existence of a Cu transport system. Bacterial Cu transport has been studied mainly in the context of metal resistance. An outer membrane protein with similarity to NosA/OprC is not part of those transport systems (107). The dual roles of NosA/OprC in pore formation and Cu binding for Cu transport and for N_2O reductase biosynthesis are still not well understood.

A mutation in the signal peptide of NosZ results in a cytoplasmic enzyme that lacks Cu. The metal can be inserted in vitro into the Cu_A site, indicating that at least for Cu_A, the lack of Cu is not due to misfolding but lies in the incompetence of the cytoplasmic compartment for Cu insertion (223). On the other hand, a mutation in the metal insertion complex by inactivating *nosD* does not affect N_2O reductase export (466, 581). Cu-deficient cells have a low N_2O reductase activity that can be reconstituted by exogenous Cu (542, 581). When Zn, Cd, or Ni is offered to Cu-deficient cells, these metals may become incorporated into N_2O reductase or interfere with the insertion process to produce an inactive enzyme in either case.

The biosynthesis of N_2O reductase with a functional Cu_Z center requires the periplasmic NosD protein and energy at an undefined step. The energy requirement is inferred from the potential ATP/GTP-binding properties of NosF and represents a further parallel to bacterial permeases. There, the transport process is driven by the activity of the ABC ATPases. NosF shows a sequence relatedness to the ATP-hydrolyzing proteins of the ABC transporters (357, 984). The structural characteristics of a large family of proteins that catalyze ATP-dependent reactions are found in the NosF protein (946).

The *nosD* gene encodes a hydrophilic protein with a signal peptide. A putative function of NosD is that of a Cu insertase, but alternative functions are feasible (980). C-terminal proteolytic processing, as shown for Ni incorporation into hydrogenase, appears not to take place with NosZ, which deviates in electrospray mass spectrometry only by 41 Da from the predicted mass of the mature protein (223). Periplasmic binding proteins have been typed into various classes. NosD, underlining its special nature, does not fit any of them (807). NosD has no notable sequence motif that could indicate a site of Cu coordination. The components of the insertion complex of *P. stutzeri* are not related by sequence similarity to Cu transport and resistance components of the pseudomonads (107). If NosD has an insertase function, it is not known from where it receives the Cu.

Copper homeostasis of the bacterial cell has to bridge the metal requirement for vital copper-containing proteins on the one hand and avoidance of the toxic effects of copper on the other hand. The intricate mechanism of copper processing for N_2O reductase emphasizes the fact that copper is moved along strictly regulated pathways, beginning at the outer membrane and targeted to acceptor apoenzymes, metal-binding components, storage and detoxification proteins, and ion pumps of the import and export pathways.

TOPOLOGY OF THE DENITRIFICATION SYSTEM

Arrangement of the Denitrification Apparatus across the Inner Membrane

The organization of denitrification components in and on both sides of the cytoplasmic membrane of a gram-negative bacterium is shown in Fig. 27. The membrane-bound enzymes are the respiratory nitrate reductase and NO reductase. Nitrite reductase, the key enzyme of denitrification, is periplasmic, as are N_2O reductase and the soluble dissimilatory variant of

nitrate reductase, NapAB. The membrane-bound components required for denitrification, including the ancillary systems, comprise as many as one-third of the genes of the *P. stutzeri* 30-kb denitrification cluster (Fig. 4).

The topological picture has been achieved by applying a number of complementary methods which include measurement of proton stoichiometries based on assumptions of the chemical reactions, detection of enzymes and electron carriers on fractionation of the cell compartments, and immunocytochemical investigation of enzyme location in situ. More recently, evidence was added from the analysis of DNA-derived amino acid sequences, signal sequences for protein export, and predictive methods for the topology of membrane proteins. In a few instances, reporter gene fusions have provided topological information, which for the majority of membrane-bound or exported denitrification components remains to be fully exploited.

The combined available evidence leads to the arrangement of the denitrification system depicted in Fig. 27. The model applies to *P. stutzeri* but has been developed along similar lines for *Paracoccus denitrificans* (795, 869). The aerobic respiratory chain of *P. stutzeri* is presumed to be similar to that of *P. aeruginosa*, which comprises primary dehydrogenases, coenzyme Q-9, the cytochrome bc_1 complex, cytochrome c_{551}, and more than one terminal oxidase (954). A *cb*-type (formerly *co*-type) oxidase and a cyanide-insensitive oxidase have been isolated from *P. aeruginosa* (169, 548, 936). The cyanide-insensitive oxidase is probably a quinol oxidase (669). Evidence from gene amplification indicates that *P. aeruginosa* has the capability to encode a cytochrome cbb_3-type oxidase (*ccoN* homolog) (817). Of the terminal oxidases of *P. stutzeri*, a cytochrome *cb* complex has been partially purified (329). Again, a *ccoN* homolog encoding cytochrome cbb_3 is present (883). For both pseudomonads, the relationship of the isolated cytochrome *cb* oxidase with the *ccoN* gene has to be investigated.

Periplasmic constituents. The cytochrome cd_1 nitrite reductases from *P. aeruginosa*, *P. fluorescens*, and *P. stutzeri* (159, 466), the Cu-containing nitrite reductases from "*A. cycloclastes*" and *Alcaligenes xylosoxidans* (159), and the N_2O reductase from *P. stutzeri* (466) were all shown to be periplasmic by direct immunocytochemical location. Prior to the in situ evidence, both types of nitrite reductases were found in the periplasm on fractionation of the soluble cell compartments of *P. aeruginosa* (916), *P. stutzeri* (581), *Rhodobacter sphaeroides* IL106 (719), and *Paracoccus denitrificans* (8). Proton consumption by *Paracoccus* cytochrome cd_1 involves the outer but not the inner side of the cytoplasmic membrane, in conformity with the periplasmic enzyme location (93).

Part of cytochrome cd_1 appears to be associated with the membrane. Relatively strong binding of cytochrome cd_1 to the cytoplasmic membrane from *P. aeruginosa* has been noted (929). A membrane-associated nitrite reductase activity of *P. stutzeri* was tentatively attributed to cytochrome cd_1 (982). Cytochrome cd_1 of *Thiobacillus denitrificans* exists both as a soluble enzyme and as a species that can be solubilized by Triton X-100 (353) or acetone (375). In *Halomonas halodenitrificans*, the enzyme sediments with the membrane fraction and is solubilized therefrom by detergent (532). The mode of anchoring cytochrome cd_1 to the membrane has not yet been addressed. One possibility is the association with the membrane-bound NO reductase.

For the export to the periplasm, both types of nitrite reductases are synthesized as precursors with N-terminal signal sequences. The precursor sequences exhibit the standard structural features of signal peptides for *sec*-dependent protein translocation (144, 430, 608, 770). The secretory system *sec* of *E. coli* recognizes heterologous signal peptides of denitrification components and exports the azurins of *P. aeruginosa* (137, 438) and *Alcaligenes denitrificans* (351) and the pseudoazurin (928) and CuNIR (608) of *Alcaligenes faecalis* S-6 into the periplasm. These observations and the general features of the signal peptides allow the conclusion that *sec* systems are operative in the source bacteria of these genes. The precursors of the periplasmic electron carriers cytochrome c_{550}, cytochrome c_2, azurin, pseudoazurin, and a number of other precursor proteins (pre-NirB, pre-NirN, pre-NirC, pre-NosD) have the characteristics of signal peptides. Their functionality is not proven in each case. The export process may not in each case be obligatorily dependent on a signal peptide, since that of cytochrome c_2 can be removed without affecting protein translocation (98).

The periplasmic location of N_2O reductase was first inferred from the proton consumption balance, which indicated that protons for N_2O reduction are provided from the outside and not from the cytoplasmic side of the membrane (92). N_2O-reducing activity was later found in the periplasmic cell fraction of *Paracoccus denitrificans* (9, 91) and *Rhodobacter sphaeroides* (852), and consequently the periplasm of *Rhodobacter capsulatus* served as the source for the isolation of the enzyme (553). Electroimmunoassay of cell fractions (581) and immunogold staining (466) provided compelling evidence for the periplasmic location of N_2O reductase from *P. stutzeri*. Membrane-associated variants of N_2O reductase exist in *Flexibacter canadensis* (421) and *Thiobacillus denitrificans* (353). The enzyme from the latter source exhibits properties similar to that of the soluble N_2O reductase.

The gene-derived amino acid sequences indicate that N_2O reductases are synthesized as preproteins for protein translocation. The signal peptides of the NosZ proteins from *P. stutzeri* (223, 974) and *Paracoccus denitrificans* GB17 (68) comprise about 50 residues, which is unusually long and more typical of gram-positive bacteria. Equally long peptides can be deduced from the *nosZ* genes of other sources. Signal peptides usually have no conserved primary structures, but those of the NosZ proteins have been found to share with hydrogenases a conserved motif centered around a pair of arginine residues (974). Mutation of the first arginine of the pair causes NosZ to accumulate as the precursor protein in the cytoplasm. At the same time, the enzyme is devoid of Cu, indicating that not only is the arginine residue essential for NosZ export but also that translocation of the protein occurs before or concomitant with Cu insertion. Besides N_2O reductases and hydrogenases, a twin-arginine motif in a structurally conserved environment is present in the signal peptides of a variety of mostly periplasmic, sometimes also membrane-bound enzymes requiring cofactor insertion or processing. The group includes polysulfide reductase, iron oxidase, periplasmic nitrate reductase, methylamine dehydrogenase, and trimethylamine *N*-oxide reductase. From the mutational evidence and the distribution of the motif among proteins requiring posttranslational processing and maturing, we have proposed that it may be a recognition sequence as part of a specialized transport and/or processing event (223).

A comprehensive survey of proteins that exhibit the twin-arginine motif has found it in proteins with polynuclear Cu sites, iron-sulfur centers, a molybdenum cofactor (MGD or MPT cytidine dinucleotide), tryptophan tryptophylquinone, or flavin adenine dinucleotide. All proteins of the family have the arginine pair within the consensus sequence (S/T)-R-R-X-F-L-K. It has been speculated that a leader peptide with this feature acts as the targeting signal for the periplasm and becomes operative only after a cytoplasmic cofactor has been inserted into the preprotein (67). Current evidence, however,

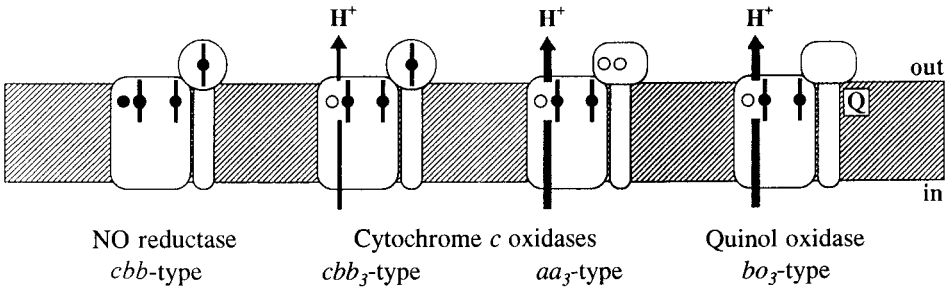

FIG. 28. Common structural motifs and their variations in NO reductase and heme-copper oxidases. Where more subunits form the oxidase complex, the models are reduced to the two homologous subunits. Apart from the quinol (Q)-oxidizing cytochrome bo_3, the oxidases function with *c*-type cytochromes as electron donors. Single open circles, Cu_B sites; pair of circles, Cu_A site; full circles, Fe atoms; short bars, heme groups; arrows, proton-translocating property. For explanations see the text. Reproduced with permission from reference 863.

does not favor a cytoplasmic Cu insertion for N_2O reductase (223, 980). The effect of the twin-arginine motif on protein transport has been experimentally addressed previously with hydrogenase and imported chloroplast proteins, where in both instances it was found that an arginine cannot be substituted by another positive charge (130, 609). This emphasizes its role as a putative recognition sequence.

Disulfide bonds have been found in N_2O reductase (224) and azurins (5, 614) and may yet be found in other denitrification components. These bonds must be introduced into the functional forms of the proteins, which is likely to occur in the periplasm by a disulfide isomerase as part of the final folding process after the proteins have been exported. The general principles of periplasmic protein folding are emerging from studies with *E. coli*. They can be assumed to be largely valid for other gram-negative bacteria. Protein disulfide isomerases are clearly an important component of the folding and maturation processes of periplasmic proteins (924).

NO reductase, an integral membrane protein. From the topological situation in gram-negative bacteria, it is clear that most of the denitrification process is a periplasmic event. With NO being generated in the periplasm, its reductase could also be a soluble component, akin to the reductases for nitrite and N_2O. Since there is no evidence that NO is a proton pump, which would provide a compelling rationale for its membrane-bound nature, possible explanations can be derived from evolutionary considerations and/or the function of NO reductase as a protective measure.

The location of NO reductase in the inner membrane indirectly protects cytoplasmic components against unwanted reactions with NO by providing an NO sink at the cell envelope. A membrane does not represent a permeability barrier for NO, and there is a preferred transport of NO into the lipid bilayer, the extent of which depends on the membrane composition. The cross section of a membrane has been elegantly probed by placing a nitroxide spin label at different positions of the acyl side chain of stearic acid (798). The NO concentration within the membrane is monitored by EPR line-broadening on collision of NO with the spin label, whereby the collision rate is proportional to the product of NO concentration and the NO diffusion coefficient. An important result of this technique is evidence for the increased diffusion concentration of NO in the hydrocarbon region of the membrane with respect to the zone of the polar head groups of the lipids and the aqueous phase (798). Preferred transport of NO into the lipid bilayer is an appealing hypothesis for having NO reductase embedded in the membrane with the catalytic site in the lipid phase. Efficient catalysis and cellular protection can be viewed, therefore, as the complementary aspects of the same physical parameter. The role of the hydrophobic NorE protein or its homologs may be related to this aspect. If this protein is indeed associated with the NorCB complex, its role could be to provide a guidance element for NO toward the catalytic subunit of the reductase.

Although NO reductase has structural similarity to COX, apparently it does not pump protons. Most of the residues proposed for the vectorial proton movement in the aa_3-type oxidase are not conserved in NO reductase. This, however, is largely also the case for the cytochrome cbb_3 oxidase, to which NO reductase is most closely related and which pumps protons, although with low efficiency (Fig. 28) (187, 666). The most weighty arguments against NO reductase as a proton pump are the location of the proton-consuming site at the outer site of the cytoplasmic membrane (92, 559, 744) and lack of buildup of a membrane potential when electrons are supplied from the periplasmic side to NO reductase (59).

NorB has sufficient sequence similarity to terminal oxidases to include NO reductase within the greater family of heme-copper oxidases. The possibility to model NorB on the peptide fold of COX I strongly supports this notion (Fig. 19). Membership in the oxidase superfamily is defined by the existence of a subunit homologous to COX I of mammalian COX that carries a binuclear heme-copper center. A current survey reveals about 80 members within this family, divided into five subclasses of which two are quinol oxidases (272). Demonstration of the COX-III homolog (NorE) as part of NO reductase would make the relationship with COX even closer.

NO reductase and the cbb_3-type oxidase FixNOQP of the heme-copper oxidase family are seen currently as primitive members of an evolutionary development in which electron donation to the catalytic subunit of the oxidase occurred initially by a *c*-type heme but later replaced by the Cu_A center that was lost again in the quinol oxidases (Fig. 28). Indeed, a Cu_A domain is still recognizable in the primary and tertiary structures of the *E. coli* quinol oxidase (864, 908).

In the evolutionary debate concerning the occurrence of denitrification relative to aerobic respiration, Broda and Egami have been the exponents of opposite positions. Broda has argued that the evolution of denitrification followed that of the aerobic electron transport chain (104). Egami proposed a sequence of events where denitrification preceded oxygen respiration (230). The lack of N oxides in the early Earth atmosphere has always been a strong argument against an early development of denitrification. This argument lost its weight by the demonstration of N oxide formation due to lightning and meteorite impacts (for example, see references 440, 534, and 952). Formation of NO is predicted for both CO_2-rich and CO-rich atmospheres, with the former also generating a sig-

nificant amount of N_2O (242). Removal of this principal obstacle led Mancinelli and McKay to support Egami's hypothesis that denitrification arose prior to aerobic respiration (534). NO could have been oxidized by various mechanisms (534, 952) and ended up in the early oceans as nitrate and nitrite to provide the substrates for other denitrification enzymes also. The electron transfer to nitrate requires only a very short chain of a dehydrogenase coupled via the quinone pool to the reductase (Fig. 27). Crediting the above hypotheses and recent findings, it is feasible that N oxide reductases preceded oxygen reductases. The possibility of an evolutionary relationship of the Cu_A domains of COX and N_2O reductase had been suggested on the basis of their structural similarity (965, 974). Given the geochemical situation, it is conceivable that NO reductase or its precursor is a link in the evolutionary line toward proton-pumping oxidases (714, 863). The precursor of the NorB subunit of NO reductase may have provided the polytopic scaffold core for the catalytic subunit of the oxidases without acquiring proton-pumping capability itself. NO enrichment in the membrane may later have provided the selective pressure to retain NO reductase in the membrane.

Gram-Positive Denitrifiers

Gram-positive bacteria lack the periplasmic compartment confined between two membranes, although the cell wall retains a certain set of proteins to give rise to what may be an operational periplasm (566). How does this group of bacteria cope with the topological constraints, and how are the enzymes arranged and anchored at the cell membrane? The various modes of anchoring a protein to the gram-positive membrane have been explored with the components of aerobic respiration. A terminal oxidoreductase can be membrane bound either by being an integral component (applicable to nitrate reductase and NO reductase) or by being anchored in the membrane by a hydrophobic transmembrane anchor. This can be an uncleaved signal peptide or a thioether-bonded acyl glycerol of a C-terminal cysteine residue. The anchoring problem also pertains to electron donors for their cognate oxidases. Aside from a protein or an acyl anchor, an electron carrier may be fused to its oxidoreductase.

Bacilli make up a sizable number of denitrifying species (965), yet only a few studies of denitrification have been directed at them. Membrane-bound respiratory nitrate reductases have been purified from *Bacillus licheniformis* (873), *B. stearothermophilus* (148), and *B. halodenitrificans* (Table 3) (449). The nitrate-reducing site is oriented toward the cytoplasm, as in gram-negative bacteria (901).

The respiratory nitrite reductase of *B. halodenitrificans* is a dimeric Cu-containing enzyme with type 1 and type 2 Cu, whose primary structure may be homologous to that of gram-negative bacteria. Nitrite reductase is firmly bound to the membrane (198); its electron donor is not known. In contrast to many gram-negative bacteria, *B. halodenitrificans* has no type 1 blue Cu protein. The enzyme does not react with *Bacillus* cytochrome c_{550} or with the blue Cu protein from "*A. cycloclastes*." The respiratory nitrite reductase from *B. firmus* is also membrane bound. From studies of proton consumption, it is assumed that its catalytic site, as well as that of N_2O reductase, is oriented toward the inside (851). A membrane-bound nitrite-reducing activity (sensitive to Cu chelation) has been reported for *B. stearothermophilus* (346).

Within the context of how the cell copes with cytoplasmic formation of NO, an NO-binding multiheme protein (64 kDa) has been discovered in *B. halodenitrificans* and proposed to exert a protective function (196). NO is bound reversibly by this protein, which may contain up to six groups of protoheme IX in the high-spin form. Denitrifying cells show the EPR features of a heme nitrosyl complex that is attributable to this heme protein.

NO is reduced by *B. cereus* (435). NO-reducing activity is membrane bound in *B. stearothermophilus* (346). Thermophilic bacilli may or may not utilize exogenous N_2O, even though they denitrify and have an N_2O reductase activity (271, 286, 346). Vigorous denitrification activity yielding N_2 from nitrate, nitrite, or N_2O was reported for *Bacillus azotoformans* (647). *Bacillus macerans*, previously reported to denitrify, ammonifies nitrite and has, at best, denitrification sensu lato (729).

Oxygen is a regulatory factor in the expression of denitrification in the genus *Bacillus* (221, 735, 751). A formate-nitrate oxidoreductase system is expressed anaerobically in *B. licheniformis* (746). The anaerobic response in these cases is likely to be mediated by an FNR-like protein (see the following section).

REGULATION

The dominant exogenous signals that induce the synthesis of the denitrification systems are low oxygen tension and the presence of a respirable N oxide. Metal ions are also expected to elicit regulatory responses. Fe, Cu, and Mo should directly or indirectly affect the biosynthesis of the denitrification components that depend on these metals. A bacterium switching to denitrifying conditions has a sudden demand for iron. The heme and nonheme Fe-containing enzymes and many cytochrome electron carriers have to be synthesized de novo upon the shift to anaerobiosis and expression of the denitrification system. A study of how external Fe availability or mobilization from internal sources may influence the expression of the denitrification system is lacking.

The outer membrane protein NosA is regulated by Cu. Copper deficiency affects the Cu_A content of *Paracoccus denitrificans* cytochrome aa_3 and the proportion of hemes A and A_3 (367). The Cu concentration in the medium influences cytochrome cd_1 synthesis by *P. aeruginosa*; i.e., supplementing the culture medium with Cu increases the cellular content of cytochrome cd_1 considerably (930). The underlying process has not been investigated. The ramifications of the requirement of Cu and Mo for denitrification have been covered in the preceding sections.

Systemic Responses to Oxygen and N Oxides

In principle, the exogenous parameters oxygen and nitrate oxides could act via two transcription factors mediating the oxygen and the N oxide response and each step of the denitrification process could be under the dual control of these factors. A first set of regulatory genes has been discovered, and it became immediately clear that a view of only two global regulators controlling denitrification is a simplistic one. Members of the same regulator family perform different functions in distinct genetic backgrounds and with different respiratory systems as their targets in regulating the denitrification process.

Complete denitrification should be viewed as the modular assemblage of four partly independent respiratory processes (Fig. 29). The systems for the reduction of nitrate and N_2O have the largest degree of independence and can function as autonomous respiratory processes even in complete denitrifiers. Both processes are able to respond independently of the rest of denitrification to special regulators. In contrast, nitrite reduction and NO reduction are controlled interdependently at both the transcriptional and enzyme activity levels, presum-

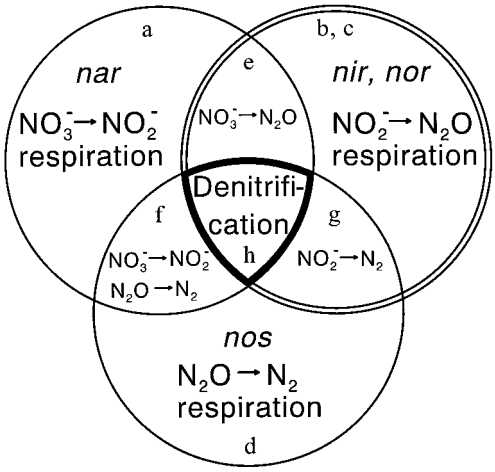

FIG. 29. Modular organization of denitrification. Four modules representing the respiratory systems utilizing nitrate (a), nitrite (b), NO (c), and N_2O (d) constitute the overall process. Complete denitrification (h) is achieved only when all four modules are activated. The *nir* and *nor* regulons are coupled by a common regulator of the FNR family. Pairwise overlaps (e to g) of the individual respiratory modules occur naturally in denitrifying or other N oxide-utilizing bacteria.

ably to prevent NO accumulation. A hierarchical control circuit targeting both reactions simultaneously is required in this instance.

As the third regulatory element, global control factors have to ensure the expression of the complete pathway for a coordinate regulation of the denitrification process. As depicted in Fig. 29, complete denitrification takes place only when the four modules constituting the process are expressed and function concomitantly. Figure 30 shows several emerging regulatory variations and the current situation will be discussed below.

FNR-like regulators. FNR-like factors have been recognized as important elements of the regulatory network of denitrification. FNR is a *trans*-acting protein of *E. coli* that activates gene expression under anaerobic conditions and is structurally related to CRP, the cAMP receptor protein, synonymous with catabolite activator protein (307, 786, 844). The name FNR reflects the regulatory role of this factor in fumarate and nitrate reduction. FNR has been modeled on the crystal structure of CRP (Fig. 31). It discriminates a palindromic TTGAT-N_4-ATCAA motif, centered at a preferred distance of −41.5 nucleotides from the transcription start of positively regulated promoters, although greater distances are also permitted when there is a topological rearrangement of the RNA polymerase, the C-terminal domain of its α subunit, and FNR (909). The recognition motif is usually referred to as the FNR box and sometimes also as the anaerobox (Table 10). Depending on the location of this box, FNR can act as either an activator or a repressor. For a few unidentified gene products, aerobic regulation by FNR has been reported (307, 722).

The following properties are relevant for a particular group of FNR-like factors involved in denitrification. FNR is an extremely labile Fe-S protein and is isolated mainly in its apoform. When purified from a mutant predisposed for protein dimerization by effecting an Asp154-Ala substitution, it shows an EPR signal of a $[3Fe\text{-}4S]^+$ species (450). Isolation of such a mutant was paramount for the identification of FNR as an Fe-S protein. The in vitro reconstitution of FNR by NifS protein yields a $[4Fe\text{-}4S]^{2+}$ species. Enhanced DNA binding and activation of transcription is found with this species (297). The $[4Fe\text{-}4S]^{2+}$ cluster is coordinated either within the peptide chain, probably by a set of essential cysteines at positions 20, 23, 29, and 122 (561, 747), or by both subunits of the dimer with cysteines contributing from either peptide to bind the cluster at the subunit interface (Fig. 31) (297). The set of cysteines is conserved in many but not all members of the FNR proteins (784).

The question how the O_2 level or a related redox signal is transduced to and processed by FNR is subject to hypotheses involving the redox chemistry of Fe, reversible Fe binding, a change in the quaternary state, protein modification, or interaction with a redox effector (307, 845). An assembly/disassembly mechanism of [4Fe-4S] cluster has been advanced as a model for O_2-dependent interconversion of active and inactive forms of FNR (56, 451). Iron cluster formation and protein dimerization are essential features for the activity of FNR (298, 506, 507, 562). Dioxygen can provide the signal for FNR activity without the need for a special signal transfer pathway (49). Azide as an O_2 analog does not affect FNR function in *E. coli*. Mutations in the respiratory complexes do not affect FNR functionality, and respiration itself does not exert a protective effect on FNR. FNR-regulated genes show half-maximum expression around 1×10^3 to 5×10^3 Pa of oxygen in the medium, which is thought to represent the internal pO_2 also

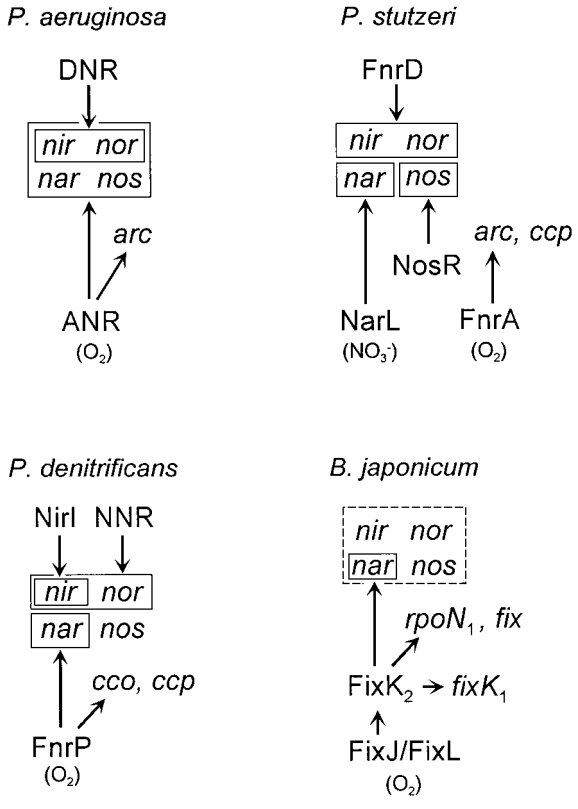

FIG. 30. Positive regulatory circuits controlled by different types of transcription factors in denitrification. Arrows point to boxed groups of target genes or individual operons under the control of a given regulator. The exogenous signals oxygen and nitrate are shown in parentheses where they are correlated with a particular regulator. Structurally and functionally homologous transcription factors are DNR, FnrD, and NNR; structurally but not functionally homologous factors are ANR, FnrA, and FnrP. It is not known whether $FixK_2$ controls only *nar* genes or the entire denitrification apparatus in *Bradyrhizobium japonicum* comparable to the action of ANR. Genetic acronyms: *arc*, arginine catabolism; *cco*, cytochrome cbb_3 oxidase; *ccp*, cytochrome *c* peroxidase. For a discussion, see the text.

TABLE 10. Structural elements and specificity of coexistent FNR-like factors found in denitrifiers

Source and factor	Class	Sequence of recognition helix[a]	Gene with FNR box	Sequence of recognition motif[b]	Distance from transcription start	Reference(s)
P. aeruginosa						
ANR	FNR	SRNEIGNYLGLAV**E**TV**SR**VFTRFQ	*arcD*	ctaTTGAcgtggATCAgc	−39.5	264, 963
			nosZ	caacTGATtcccgcCgtc		974
			narG	?		942
			anr	None		963
DNR	FixK	AKQLVAGHLSIQP**E**TF**SR**IMHRLG	*nirS*	atcTTGATtccggTCAAg		19, 22
			nirQ	tgcTTGAccggaATCAAg		21
			norC	atcTTGATtgccATCAAg		23
			dnr[c]	agcTTGccgtgcgTCAAg		22
P. stutzeri						
FnrA	FNR	SRNEIGNYLGLAV**E**TV**SR**VFTRFQ	*arcD*, *ccp*	?		173, 882
			fnrA	None		173
FnrD	FixK	AKQLVAGHLSIQP**E**TF**SR**IIRRLN	*nirS*	ctcTTGATtgccgTCAAg	−43.5	430, 882
			norC	ttcTTGATtgccATCAAg	−40.5	968
			nosR[d]	gcgaaGATggaaATCAAg	−137.5	171
			nosZ (P3)[d]	cagTTGATccccgTgcAa	−52.5	171
			fnrD[c]	actTTGAcgatcATCAAg	−51.5	882
P. denitrificans						
FnrP	FNR	TREAMADYLGLTL**E**TV**SR**QMSALK	*ccoN*	agaTTGAcgcagATCAAa		187, 871
			narG	?		
			fnrP[c]	accTTGAcccaaATCAAa		
NNR	FixK	NKRLIAGHLGMQP**E**SL**SR**AFARLR	*nirS*	gccTTaAcaaaggTCAAa		185, 870
			nirI	gctTTGAcctttgTtAAg		185
			norC	cacTTGActttcATCAAt		186
			nnr	None		870
R. sphaeroides						
FnrL	FNR	TREEMADYLGLTL**E**TV**SR**QVSALK	*hemZ*	cgcTTGATctgaATCAAa		955
			fnrL	gctTTGATtcagATCAAg		
NnrR	FixK	TRQNISEMTGTTLHTV**SR**LLSAWE	*nirK*	ttgTTGcgcaaccgCAAa	−43.5	832, 833
			norC	tctTTGtgatcccgCAAc	−42.5	39
			nnrR[c]	tcaTTGtgctgccgCAAa		833
B. japonicum						
$FixK_1$	FNR	SRQDIADYLGLTI**E**TV**SR**TFTKLE	?			15
$FixK_2$	FixK	CRRDIGDYLGLTL**E**TV**SR**ALSQLH	*fixN*	atcTTGATttcaATCAAt		249, 656
			fixG	cgtTTGAgctggATCAAc	−39.5	657
			$fixK_1$	gaaTTGATctgggTCAAc		15
			$fixK_2$	None		249
E. coli						
FNR[e]	FNR	TRGDIGNYLGLTV**E**TI**SR**LLGRFQ α_E α_F	Consensus	a--TTGAT--a-ATCAAt	−41.5	307, 786
			fnr[c]	aaaTTGAcaaatATCAAt		

[a] Residues making contacts with DNA are boldfaced.
[b] Identities with the FNR box are uppercased.
[c] Recognition motif for autoregulation or a hierarchical FNR-like factor.
[d] The factor controlling the expression of this gene is unknown.
[e] The recognition helices α_E and α_F are underlined.

(49). The value is equivalent to about 0.5 to 2.4% air saturation.

FNR-like factors are found in a variety of gram-negative and a few gram-positive bacteria. They show structural and functional variations which group them into several classes (249, 307). The prototype of the FNR group (which is also divided into class I*A* and I*B* according to the spacing of the N-terminal cysteines) is FNR from *E. coli*. The FNR-like factors ANR, FnrA, FnrL, and FnrP of denitrifiers belong to this group. The FixK group (or class I*C*), named after its first such member, lacks the N-terminal cysteine cluster (42). FNR from *B. subtilis* also lacks the N-terminal cysteines but instead has four such residues in the C-terminal domain and two located centrally, making an Fe-S center feasible (168). *Bacillus* FNR is the first example within the FNR family that has such a C-terminal location of a putative cysteine cluster.

With the structures of denitrification genes forthcoming, the existence of FNR boxes in the promoter regions at critical distances to transcriptional start sites (Table 10; Fig. 4) suggested a molecular rationale for the oxygen control and expression of individual reductases. Reporter gene fusions have demonstrated anaerobic regulation of the promoters of *nirS* (19), *nirQ* (21), and *nosR* (171). The FNR boxes of denitrification genes are centered at positions −40 to −50 relative to the transcriptional start (Table 10). In *nosR*, an FNR box is located at an exceptional distance of −137.5. FNR motifs were also described for the promoter regions of the genes for azurin

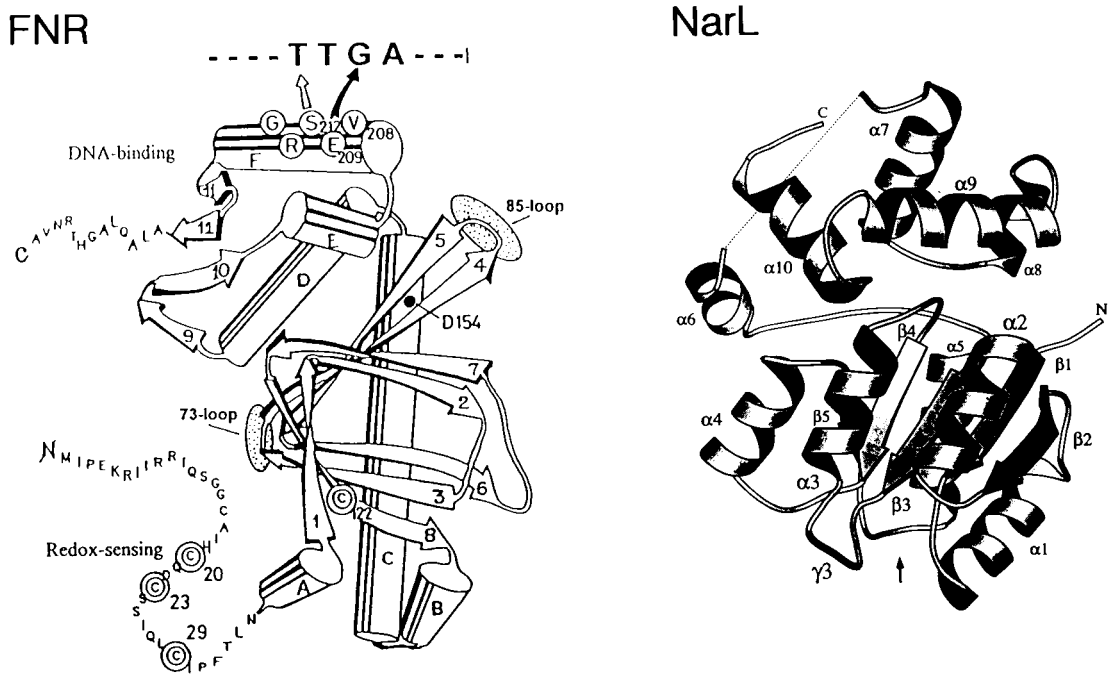

FIG. 31. Structural model and X-ray structure of the transcription factors FNR and NarL. (Left) The FNR model is based on the crystal structure of CRP. The model of FNR shows the DNA contact site to the cognate TTGA sequence, the 85- and 73-loops binding to RNA polymerase, the conserved cysteine residues (circled) of the [4Fe-4S] cluster, and the target site Asp154 for generating the FNR* mutant. Reproduced with permission from reference 306. (Right) In the NarL structure, the C-terminal part (top) is the DNA-binding domain. Its link with the N-terminal domain (bottom part), which was not resolved in the electron density map, is shown by a dotted line reaching from helix α7 to helix α6. The arrow points to the loop with Asp59 that is the phosphorylation site to generate NarL-P. Reproduced with permission from reference 33.

(352) and CuNIR (608, 941). *nosZ* is regulated anaerobically, although its FNR recognition motif is notably degenerate. It is possible that only a half-site is functional; also, analysis of the *nirB* promoters has indicated that not every potential FNR box is active (298). The participation of an FNR factor in *nosZ* regulation requires further proof; an additional complication is the involvement of the NosR protein.

A promoter probe vector carrying *lacZ* under the control of an FNR recognition motif first indicated the existence of FNR-like elements in *P. aeruginosa* (519) and *Paracoccus denitrificans* (783). Arginine degradation allows *P. aeruginosa* an anaerobic way of life in the absence of fermentative capabilities. In searching for the regulator for the system of arginine degradation encoded by the *arcDABC* genes, the Nar^- mutant S1239 (a relict from the initial period of genetic analysis of denitrification [866]), was found to be pleiotropic with respect to nitrate and arginine utilization. Investigation of the affected locus led to the discovery of *anr* (264). ANR is a homolog of FNR (51% sequence identity) that functionally replaces FNR in *E. coli*, for example, in activating the *pfl* genes for the pyruvate formate-lyase complex (721, 963). With its N-terminal and central cysteine residues, a DNA-binding motif, and the glycine-rich β-roll structure, ANR conserves essential structural features of an FNR protein. It was named ANR to reflect its role in arginine catabolism and nitrate reduction, because *P. aeruginosa* has no fumarate respiration.

The promoter region of the *arcDABC* operon carries at position −39.5 the nucleotide sequence TTGAC-N_4-ATCAG, resembling the FNR box (Table 10). Manipulating this sequence decreases or abolishes the expression of the *arc*-encoded enzymes (264). Most importantly, deletion of *anr* affects all enzyme activities of the four steps of the denitrification pathway (942). ANR thus features as a global regulator in *P. aeruginosa* for anaerobic metabolism that, in addition to controlling the *arc* operon and other metabolic activities like cyanogenesis, regulates the entire denitrification process. The details of how this control is achieved remain to be explored.

The discovery of ANR meant a breakthrough in the understanding of the anaerobic regulation of denitrification. With the evidence for an FNR-like transcription factor and demonstration of its role in denitrification, a fundamental aspect of oxygen control seemingly had been solved. Indirect evidence from DNA-DNA hybridization and the use of an *arcDA'-'lacZ* fusion vector suggested that ANR was also present in other pseudomonads (721, 963). It was expected that the properties of ANR would provide the paradigm for other denitrifiers. However, it soon became apparent that anaerobic regulation in denitrifiers is more complex than depending on just a single FNR-like regulator (Fig. 30).

In searching for the regulator of anaerobic gene expression in *P. stutzeri*, an important finding was made. With *anr* as the screening probe, the homologous *fnrA* gene was isolated (173). The FnrA protein is 86.1% identical to ANR and 51.2% identical to FNR. An *fnr* terminology was chosen to account for different alleles and underline the fact of membership in the FNR family, even though *P. stutzeri* is not known to respire fumarate. Although *fnrA* complements an *E. coli fnr* mutant for growth on minimal nitrate-glycerol medium, it unexpectedly exerts no effect on denitrification. The activities and protein levels of all four reductases are preserved in an *fnrA* mutant. On introduction of an FNR promoter probe vector into the *fnrA* mutant, anaerobically induced FNR activity remained clearly detectable. This led to the proposal of the coexistence of more than one FNR-like factor within the same denitrifying

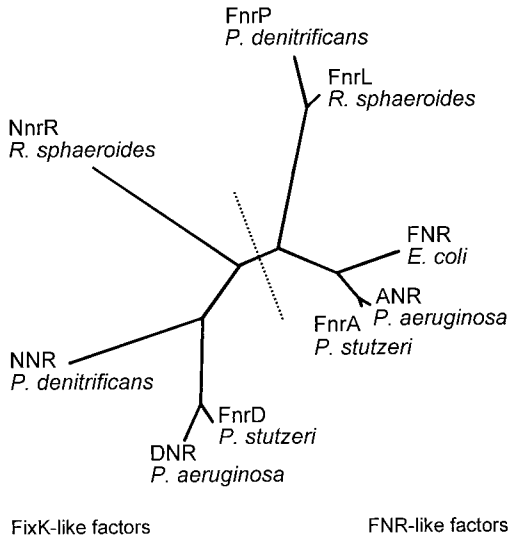

FIG. 32. Sequence relatedness among FNR homologs found in denitrifiers. The dashed line separates regulators carrying the N-terminal cysteine cluster (FNR-like factors) from those without it (FixK-like factors). The tree was constructed with CLUSTAL W and PHYLIP software (Fig. 3). For sequence sources, see Table 2.

bacterium (173), a finding that was concurrently established for diazotrophs (15, 249).

On sequencing the flanking regions of *norCB* operons, a set of *fnr* homologs was discovered: *nnr* of *Paracoccus denitrificans* (870), *dnr* of *P. aeruginosa* (22), *nnrR* of *Rhodobacter sphaeroides* (833), and *fnrD* of *P. stutzeri* (882). In a so far unique case of several *fnr* genes within the same bacterium, a third *fnr*-like gene exists in *P. stutzeri* as part of the *narXL* region (320). The distinguishing structural element of all these regulators is the lack of the cysteine cluster, which places them into the FixK-like group. A further member is $FixK_2$, which controls nitrate respiration of *Bradyrhizobium japonicum* (249). The genes for denitrification of this bacterium have not been characterized to allow conclusions of the location of $fixK_2$ relative to the *nir* and *nor* genes. It is remarkable that the currently known FixK-like factors involved in denitrification are in tandem, although not necessarily cooperative, with a member of the FNR group. In addition to ANR and FnrA, these are FnrP of *Paracoccus denitrificans* (871), $FixK_1$ of *B. japonicum* (15), and FnrL of *R. sphaeroides* (955). Although FnrL has been identified in the nondenitrifying strain 2.4.1 of *R. sphaeroides*, it is anticipated to exist in the denitrifying strain 2.4.3 also.

The relatedness of the primary structures of the tandem FNR factors shows considerable divergence both within the same group and between the groups (Fig. 32). Low overall sequence conservation is not uncommon. A phylogeny of the pairs of FNR-like factors from a common ancestor within the same organism is not suggested. The sequence E--SR in the recognition helix that makes contact with T · A and G · C base pairs of the recognition motif is of primary significance among the criteria that establish an FNR homolog (Table 10). This sequence is conserved in nearly all FNR factors irrespective of their class and phylogenetic distance. Exceptions are NnrR from *Rhodobacter sphaeroides* and FNR from *B. subtilis*, where only serine and arginine among the specificity-conferring residues are present. The congruence of structural features of recognition helices and recognition motifs establishes a credible pattern to include the above-described FNR- and FixK-like factors from denitrifiers in the greater FNR family.

ANR affects all steps of denitrification in *P. aeruginosa*, whereas its homolog, FnrA, affects none directly in *P. stutzeri* (Fig. 30). FnrP of *Paracoccus denitrificans* acts only on nitrate reduction but not the other reaction systems (871). All the factors belonging to the FixK group, i.e., DNR, NNR, NnrR, and FnrD, act on both the *nir* and *nor* genes. $FixK_2$ of *Bradyrhizobium japonicum* affects nitrate respiration, which implies action on the *narGHJI* operon (unpublished data cited in reference 249). Whether other denitrification genes are controlled by this regulator is unknown. In contrast to *B. japonicum*, FixK of *S. meliloti* is not required for nitrate respiration (42).

FNR of *E. coli* complements an *anr* mutant of *P. aeruginosa* in activating anaerobic nitrate utilization but not arginine catabolism. In turn, the promoter of the ANR-dependent *arc* genes is not active in wild-type *E. coli*; i.e., FNR does not recognize the *arcD* promoter. ANR appears to be less stringent in its DNA-binding specificity than FNR (912). While this provides a rationale for differential activity on exchanging FNR factors between different hosts, it cannot explain how cognate genes are discriminated in the simultaneous presence of two or more FNR factors within the same cell. Recognition helices and recognition motifs reveal only subtle differences to explain the correspondence of an FNR factor to a distinct set of target promoters. Future answers may be found in a participation of neighboring nucleotides in the recognition process, as shown for FNR of *E. coli* (298), the exertion of hierarchical control over the FixK-like factors, and/or further protein requirements for transcriptional activation.

A special situation is presented by pairs of divergently transcribed genes with only one FNR box as part of overlapping promoters, such as *nirQ* ↔ *nirS* in *P. aeruginosa* and *nirI* ↔ *nirS* in *Paracoccus denitrificans*. For *nirQ* and *nirS*, it is known that both genes are regulated anaerobically (21). A mutation in *dnr* abolishes activity at both promoters and indicates that the same FNR factor exerts control over both genes from a single binding site. The divergent promoter of *hemZ* ↔ *fnrL* of *Rhodobacter sphaeroides* (*hemZ* is the *hemN* homolog in this bacterium) is perhaps designed this way to activate *hemZ* transcription and inhibit that of *fnrL* at the same time (955).

The presence of FNR boxes in DNR, NnrR, and FnrD can imply anaerobic autorepression (785) or a dependence on another FNR factor. ANR and *dnr* of *P. aeruginosa* may indeed have a hierarchical relationship, but FnrA and *fnrD* do not, since there is no effect on denitrification as a result of inactivating *fnrA*. The location of the FNR box in *nnrR* is more suggestive of negative autoregulation than of positive activation. Finally, *nnr* lacks an FNR box and can be neither autoregulated nor under the direct control of another FNR factor.

ANR, FnrP, and FnrA, as members of the putatively redox-active FNR-like group, are likely to exhibit the properties and function of the prototype from *E. coli*. FnrP positively regulates the expression of the CcoNOQP oxidase and the cytochrome *c* peroxidase and represses the quinol oxidase (871). Evidence for a role of FnrL in denitrification is not yet available but is awaited with interest, because this regulator appears to be under the hierarchical control of a particular oxidase branch of the respiratory chain (956). A mutation in the *ccoNOQP* operon between *ccoN* and *ccoO* of *R. sphaeroides* causes *hemA* overexpression under aerobic conditions. The mutant is locked in a state that signals a low oxygen level or anaerobiosis. *hemA* encodes the anaerobically functioning ALA synthase (EC 2.3.1.37) and is under the control of FnrL. Among several possibilities, it was proposed that a signal from the CcoNOQP oxidase system exerts control over FnrL (955). Since homologous *ccoNOQP* operons exist in

P. aeruginosa, *P. stutzeri*, and *Paracoccus denitrificans* it raises the prospect of similar signaling pathways there.

Within this context, it is worth recalling the puzzling observation made several years ago with *Paracoccus denitrificans* (114) and confirmed by immunochemical means with *P. stutzeri* (84) that azide causes an overproduction of respiratory nitrate reductase. In *Paracoccus denitrificans* NCIB 8944, azide and nitrite both increase the aerobic activities of nitrate reductase and nitrite reductase. The effect is not observed in the presence of chloramphenicol and has been interpreted as a regulatory signal from the respiratory chain to the expression system of the two reductases (483). In an extension of this idea, it was shown that the respiratory inhibitors azide, nitrite, cyanide, and rotenone all activate an FNR factor in *Paracoccus denitrificans* PD1222 in the absence of nitrate (484). FNR expression goes in parallel with a rise in nitrate and nitrite reductases. The conclusion is that the control exerted by the regulator depends on the electron flow to oxygen in the respiratory chain. How this status is transmitted to or perceived by the FNR factor remains to be seen.

For the anaerobic expression of denitrification genes, the seemingly resolved question of O_2 or redox sensing by an FNR factor is subjected anew to experimental scrutiny with the finding of the FixK-like factors lacking the cysteine cluster. *Bradyrhizobium japonicum* provides an example of taking advantage of a system of O_2-sensing and signal transduction in denitrification that is a main switch for the expression of genes for nitrogen fixation in bacteroids (Fig. 30). This system operates by the two-component regulators FixLJ (42, 183). FixL is a heme protein that acts as an O_2 sensor and has an altered kinase activity in response to the spin state of the heme iron (277). It activates the transcription factor FixJ, which in turn acts on *fixK*$_2$. The signal transduction toward nitrate respiration branches at this level. FixK$_2$ is hierarchical to *fixK*$_1$, *rpoN*, *fixNOQP*, *fixGHIS*, and probably the *nar* operon (15, 249). *fixK*$_1$ encodes an FNR-like factor in *B. japonicum* with unknown target genes. FixLJ systems have not been found outside N_2-fixing bacteria. This could limit this type of control to the denitrifying diazotrophs. Denitrification and N_2 fixation are antagonistic reactions in the N cycle, but this may not be the case for denitrifying diazotrophs, which can fix the N_2 derived from denitrification (228). Since denitrification provides both the substrate and ATP for nitrogen fixation, expression of denitrification in a diazotroph may be beneficial. Since the reaction proceeds rather slowly, proof is needed for a physiological significance.

Another possibility of exerting a hierarchical control over an FNR factor is represented by the *fnr*-like gene of *B. subtilis*. This gene is located between *narK* and the *narGHJI* operon (168) and is activated anaerobically by the two-component regulators ResD and ResE (599). Disruption of *Bacillus fnr* affects anaerobic growth on nitrate and abolishes the induction of nitrate reductase. ResDE is also required in *B. subtilis* for the expression of the flavohemoglobin gene, *hmp* (498). *hmp* is a homolog of *fhp* from *R. eutropha*, which affects denitrification insofar as a mutant lacking the *fhp* gene product does not evolve N_2O transiently like the wild type (162). *hmp* of *B. subtilis* requires nitrite as inducer, which hints at a more versatile N oxide metabolism by this bacterium than only nitrate respiration (280). The expression of the *hmp* gene of *E. coli* is negatively regulated by FNR under anaerobic conditions and is strongly induced by nitrite and NO. Nitrate is a weaker inducer, but both nitrite and nitrate act independently of the nitrate regulatory elements NarL and NarP (652).

The unexpected demonstration that CooA, another member of the FNR/CRP family, can accommodate a heme group in its structure (18) widens the principal modalities of redox sensing by these transcription factors, even though the CooA factor appears to be specific to CO (see below).

Interdependence of nitrite and NO reduction. The transformations of nitrite and NO to N_2O constitute a tighter regulatory and functional unit within the overall denitrification process than do the other reactions. Nitrite reduction proceeds in the cell if the further reduction of NO is ensured by an interdependent expression and activity regulation of the nitrite and NO reductases. NO is produced as a signal molecule through the activity of nitrite reductase and was proposed to act as inducer for its own reductase. Only cells that are able to produce NO express the wild-type level of NorCB (968). *nor* mutants of *Paracoccus denitrificans* shut down nitrite reduction by reducing the expression of cytochrome *cd*$_1$ and simultaneously inactivating the enzyme (186). These effects are less manifest in *P. stutzeri*, but the in vivo nitrite-reducing activity is lowered to about 10% in *nor* deletion mutants (968).

The initial observation that nitrite and NO reduction influence each other was made with mutants of *P. fluorescens* affected in *nirS*, *nirC*, and adjacent *nir* genes (938). These *nir* mutants exhibit simultaneously a low activity of NO reductase. Mutations in the nitrite reductase gene, *nirS*, or of genes involved in heme D_1 biosynthesis of *Paracoccus denitrificans* all abolish NO reduction in vivo at the same time (185). The effect on NO reductase activity of both organisms appears to be the result of a lack of enzyme expression, as shown for several *nir* mutants of *P. stutzeri*. The level of NorB is drastically reduced in comparison to the wild type, irrespective of the nature of the inactivated *nir* gene (634, 968). A mutation in *nirT* does not affect the synthesis of an in vitro catalytically competent nitrite reductase, whereas mutations in *nirFDLGH* all result in a heme D_1-less cytochrome *cd*$_1$. Since the *nir* genes exhibiting an effect on *norB* expression are organized in at least two transcriptional units, the effect has to be of an indirect nature. The common denominator behind the diverse Nir phenotypes is the lack of NO generation, which led to the conclusion that NO is required as an inducer of its own reductase (634, 968).

Evidence for the existence of a signaling pathway involving NO came from the following observation. *nirK* encoding CuNIR of *P. aureofaciens* can be expressed in its functional form in a *nirS* mutant strain of *P. stutzeri*, taking advantage of a foreign electron donor. Providing the *nirS* mutant in *trans* with an alternative NO generator by introducing *nirK* raises NorB expression to the same level as was achieved when providing the homologous *nirS* gene (968). Both the *nirS* and *nirK* gene products release NO as the signal molecule. The cell is assumed to have the means of sensing NO and converting this signal in transcriptional activity, resulting in *norCB* expression.

Regulators acting simultaneously on *nirS* and *norCB* have been found. These FNR-like factors encoded by *dnr*, *nnr*, *nnrR*, and *fnrD* form part of the *nor* regions of the respective host organisms (Fig. 2). Mutagenesis of *dnr*, *nnr*, and *fnrD* affects transcripts, expression levels, and activity of both nitrite and NO reductase (22, 870, 882). A response of the FixK-like factors to N oxides has been proposed (22, 497). *nirS* expression is nitrite dependent (20), and that of *nirK* and *norCB* responds to NO (497, 833). Cells of *Rhodobacter sphaeroides* that are unable to reduce nitrite and hence are unable to generate NO do not synthesize nitrite reductase or NO reductase.

Sensing nitrite in the periplasm requires signal transduction across the membrane. NO is formed at the cytoplasmic membrane. Because of the free diffusibility of NO, the prokaryotic two-component regulatory paradigm can be dispensed with in favor of a regulator located inside the cell that combines sensor

and activator properties. The *nirK* promoter is under the control of NnrR, which responds to NO, raising the question whether an FNR-like factor can act as an NO sensor (832).

Models for NO-sensing proteins acting as transcriptional activators are represented by the SoxR and CooA proteins. SoxR of the *sox* regulon senses NO in addition to $O_2^{\bullet -}$ (615). Among the target genes of the *sox* regulon are those for the DNA repair enzyme endonuclease IV and the Mn-containing SOD. Expression of the dismutase is part of the oxidative stress response activated in *E. coli* by superoxide anion-generating endogenous or artificial redox-cycling agents. The cognate genes are under the control of the transcription factor SoxS, which is controlled by the redox-sensing Fe-S protein SoxR (205).

The regulator CooA for the CO dehydrogenase of *Rhodospirillum rubrum* is a novel representative of the FNR/CRP family (749). The protein lacks the N-terminal cysteine cluster of FNR for redox sensing but acts as the CO sensor. Depending on the presence of CO, it binds to a consensus region, TGTCA-N_6-CGACA, that resembles the recognition motifs of CRP-regulated genes (327). The protein was overexpressed, purified, and, unexpected for this family of proteins, shown to bind heme B (18). It is easy to conceive that reacting the heme with CO would change the conformation of this factor to make it competent for DNA binding as shown in the footprinting experiments (327). A similar system can be envisaged for an NO sensor.

A close physical contact of nitrite reductase and NO reductase will ensure a limited diffusiveness of NO. Although cytochrome cd_1 is generally considered a soluble periplasmic enzyme, combined membrane-associated nitrite- and NO-reducing activities have been reported for *P. stutzeri* (982), *Rhodobacter sphaeroides* (388, 849), and *Halomonas halodenitrificans* (295). Attempts to isolate from these sources a "denitrifying" particle have not been pursued. Indirect evidence can be interpreted as the association of the two enzymes (938). The labeling of N_2O with ^{18}O from $H_2{}^{18}O$ during the reduction of ^{15}NO is about 25% in the wild type. This figure is not changed in Nos^- mutants but is reduced nearly to the background level in Nir^- mutants of *P. fluorescens*, irrespective of the affected *nir* gene. In *P. stutzeri*, transcription of *norCB* is repressed in *nir* mutants but the catalytic activity of the NO reductase is up-regulated. Whether this is also the case for *P. fluorescens* and provides an explanation for the altered pattern of ^{18}O exchange requires further study. Differential inhibitory effects of ionophores affecting the pH gradient and/or the membrane potential in *Flexibacter canadensis* have been hypothesized in terms of dissociating a nitrite-NO reductase complex (919).

The interdependence of nitrite and NO reduction is also demonstrated from mutational inactivation of certain *nor* genes and *nirQ* (186, 431). Mutagenesis of *norD* results in a phenotype similar to that of a *norQ* mutation (186). *norQ* is homologous to *nirQ*, and both are part of the *nir-nor* region. Their mutagenesis affects simultaneously nitrite reduction and NO reduction. Expression of *nirQ* in *P. aeruginosa* depends on both ANR and DNR (22). The *nirQ* product has the Walker nucleotide-binding motifs A and B (946) and shows low sequence similarity to the NtrC family of transcriptional activators (431). A C-terminal DNA-binding motif described for NirQ is not evident in the NorQ proteins. The immediate target for the NirQ/NorQ proteins within the denitrification process and their mechanism of action is unknown. *nirQ* and *norQ* have a homolog in *cbbQ* (53% identity), which is part of a gene region encoding ribulose-1,5-bisphosphate carboxylase of *P. hydrogenothermophila* (944). Coexpression of the carboxylase structural gene with *cbbQ* increased the carboxylase activity, which hints at an assembly or maturing function (unpublished data cited in reference 24).

Nitrate response. An N oxide is required for the induction of denitrification. Usually nitrate is a good inducer for all enzymes, but inducer properties have also been observed for nitrite, NO, and N_2O. The transition to anaerobiosis in the presence of nitrate induces in *Paracoccus denitrificans* nitrate reductase followed by cytochrome cd_1 activity (485). Following the transition for this bacterium at the transcript level, the same sequence of induction was observed. An anaerobic shift was not sufficient by itself to activate the transcription of the genes *narG*, *nirS*, and *nosZ* (43). The *nirS* promoter of *P. aeruginosa* has been reported to respond more strongly to nitrate than to oxygen (957). Nitrate stimulates the synthesis of denitrification enzymes of *Rhodobacter sphaeroides* IL106; in its absence, only a low enzyme level was found (574).

The induction of the overall denitrification system may be sequential or coordinate or may incorporate elements of both alternatives. In this way, the induction of a distinct reductase is possible by its cognate substrate and at the same time the joint induction of "downwardly" (in cases also "upwardly") positioned reaction systems. Nitrite acts as a strong inducer of its own reductase (467). In addition to nitrate responsiveness, the *nirS* promoter was found to be nitrite sensitive (20). Pleiotropic mutants of *P. stutzeri* defective in both the respiration and assimilation of nitrate (Nar^- Nas^- phenotype) respond to nitrite with the induction of cytochrome cd_1 (967).

The function of NO as inducer for its cognate reductase in *P. stutzeri* and *Rhodobacter sphaeroides*, in the latter concomitant with CuNIR, has been discussed above. N_2O induces both N_2O reductase and nitrate reductase. The level of N_2O reductase in the presence of N_2O is significantly lower than in the presence of nitrate (467). The question how N_2O induces its own reductase and also acts on the *nar* genes is interesting. As discussed in the context of the active site of N_2O reductase, only transition metal proteins are capable of binding N_2O. No N_2O sensor is known. Signaling might involve the anaerobic electron transfer chain (see above), but hydrophobic interactions of N_2O with the interior of a protein, inducing a change in the protein conformation (214), are also possible and must be considered. A candidate protein for the interaction with N_2O in the signaling pathway is NosR.

The expression of the various reductases in response to an N oxide has been studied in O_2-controlled chemostat culture with *P. stutzeri* (465, 467) and "*A. cycloclastes*" (160). Both a low O_2 concentration and an N oxide have to be present for the induction of denitrification enzymes. The appearance of NO reductase in *P. stutzeri* ZoBell was nearly concomitant with that of other enzymes of denitrification, indicating coordinate expression (465). This was also the case for N_2O reductase of *Paracoccus denitrificans*, which appeared concomitantly with nitrate reductase (43). Both nitrate and nitrite were found to activate the transcription of *nirS*, *nirSTB*, *norCB*, and *nosZ* of *P. stutzeri* (319). The induction of nitrate reductase occurred at a higher oxygen level than that of cytochrome cd_1, NO reductase, and N_2O reductase, suggesting that the sensory system for anaerobiosis directed at the *nar* genes is more sensitive (465, 467). The kinetics of *narH* induction preceded that of *nirS* in *P. denitrificans*, compatible with a sequential control mechanism (43). This may be rationalized in part by the fact that the *nar* genes are under the control of FnrP whereas *nirS* is controlled by NNR (Fig. 30).

Two-component sensor-regulator elements frequently control a bacterial response to environmental stimuli. Factors belonging to the regulator family of two-component systems are

just being recognized as important factors for the expression of denitrification genes. The FixLJ system was discussed above in the context of redox sensing. A *narL* homolog required for the cellular nitrate and nitrite response has been identified in *P. stutzeri* (320). The derived NarL protein has 51% sequence identity to NarL from *E. coli*. The conserved aspartate and lysine residues of response regulators are present in the *P. stutzeri* protein, as are the arginine, glutamate, and lysine residues in the DNA-binding domain of the FixJ-RcsA subfamily of transcription factors to which NarL belongs.

NarX and NarL function in *E. coli* as the sensor and regulator, respectively, responding to the external nitrate and nitrite levels under conditions of nitrate respiration (610, 791). Nitrate induces the *narGHJI* operon, *narK* gene (nitrite export), and *fdnGHI* operon (formate dehydrogenase-N) but represses the operons *frdABCD* (fumarate reductase) and *dmsABC* (trimethylamine *N*-oxide reductase) and the *adhE* gene (alcohol dehydrogenase). NarX and NarL are duplicated in the homologous factors NarQ (147, 661) and NarP (662) in *E. coli*. Certain genes are regulated only by NarL, and others are regulated by both NarL and NarP. The *napF* promoter of the operon for periplasmic nitrate reductase responds equally to nitrate and nitrite through both the NarX and the NarQ sensors. It is under the positive control of NarP and is inhibited by NarL via competition for the NarL-binding site (181).

NarL is targeted to a heptameric nucleotide sequence found by DNase I footprinting in several NarL-dependent promoters. DNA binding was shown to be affected by substitutions in the heptamer in a number of cases (512). The NarL-binding heptamer is defined as T-A-C-Y-N-M-T, where Y is C or T, M is A or C, and N is any nucleotide (842). A limited variation in this motif is allowed (215). Sequences resembling the NarL heptamer are present at various distances from the transcriptional start in the promoter regions of a number of denitrification genes including *nosZ* and *norC* (171). These motifs, however, appear not to be active, since a *narL* mutant of *P. stutzeri* is selectively affected in respiratory nitrate reductase expression but has no phenotype for the other oxidoreductases of denitrification (unpublished results).

NarL heptamers are found as single copies, inverted repeats, or direct repeats, and their position varies over a wide range, which means that they must be determined individually in nitrate-regulated promoters by footprinting and/or mutational analysis. The *narG* promoter of *E. coli* successively carries an FNR box at −41.5, five NarL heptamers in the region −57 to −101, an integration host factor-binding site around −110 to −140, and three further NarL heptamers at −185 to −208. The −195 heptamer is essential for transcriptional activation; that at −89 is required for full nitrate induction. A detailed discussion of different nitrate-sensitive promoters and their regulatory elements can be found in reference 182.

The crystal structure of NarL from *E. coli* has been solved to a 2.4-Å resolution (Fig. 31) (33). The N-terminal receiver domain with the acceptor residue Asp59 for phosphoryl transfer resembles that of the chemotaxis regulator CheY and that of the nitrogen regulator NtrC structurally. The receiver domain is a five-stranded β-sheet with three α-helices on one side of the sheet and two on the other. The DNA-binding domain consists of four α-helices, of which two form a helix-turn-helix motif. The two domains are linked by a short helix and a stretch of amino acids that is probably flexible and was not resolved in the crystal structure. Protein folding has been obtained in the closed conformation of the unphosphorylated protein, where the DNA-binding domain is folded on top of the C-terminal domain and cannot bind DNA.

narL of *P. stutzeri* overlaps 5′ with a gene that is a homolog of *narX* from *E. coli* (unpublished data). Both genes are linked to the *nar* locus, an organization reminiscent of the *nar* locus of *E. coli*, where *narLX* is adjacent to *narKGHJI*. The first demonstration of the existence of a NarXL two-component system outside *E. coli* will allow us to explore the induction of genes by nitrate and nitrite in denitrifiers. It is anticipated that the NarXL system of denitrifiers has properties similar to that of the nitrate respirer *E. coli*. Attention has been drawn above to effects of nitrate on the induction of genes for heme biosynthesis. It is very likely that the same nitrate-responsive factors are also necessary for regulating those genes.

NarX (or NarQ) constitutes the sensor protein for nitrate and nitrite in *E. coli*. The protein (≈60 kDa) has two transmembrane regions that expose a periplasmic domain of about 120 amino acids between them (907). In this periplasmic domain, a sequence element is conserved in the NarX and NarQ proteins. Mutation of Arg54 to Glu in this element abolishes nitrate sensing by NarX (129). In the absence of nitrate, NarX is believed to be in a conformation that inhibits autokinase activity and no phosphoryltransfer to Asp59 of NarL is possible. Nitrate alters the conformation of NarX to permit autophosphorylation and subsequent phosphorylation of NarL. NarL-phosphate then binds to the NarL recognition site(s) of a gene activated by nitrate. The interconvertible states of wild-type inactive NarX in the absence of nitrate and nitrate-bound active NarX are supported by mutations that lock the protein in a permanently active or inactive conformation (129).

The presence of nitrate is signaled to the *narG* operon via NarX or NarQ. NarQ also signals nitrite, and in either situation strong expression follows. The perception of nitrite by NarX results only in weak expression of the *narG* operon (662). The differential reactivity of NarX toward the two similar ligands nitrate and nitrite has been localized to the short cytoplasmic N-terminal segment preceding the first transmembrane helix (907).

How and where nitrate is bound to NarX is under investigation. A conserved periplasmic sequence element may be involved in nitrate sensing (129). Crystal structures of proteins with bound nitrate, although with no relationship to nitrate metabolism, can help to address this important question. Nitrate occupies the site of the allosteric effector chloride in *Limulus* hemocyanin (326). In the tyrosine phosphatase of *Yersinia enterocolitica*, which takes part in the cellular regulation of pathogenicity, nitrate is bound by the phosphate-binding peptide loop (238). In both cases, nitrate is hydrogen bonded with two oxygens to the N^{ε} and N^{η} atoms of an arginine residue; other hydrogen bonds extend from the oxygen atoms of nitrate to the hydroxyl group of serine in hemocyanin or to amide nitrogens of the phosphate loop in the tyrosine phosphatase. These models are important because they suggest for NarX the possibility of hydrogen bonding of nitrate to Arg54 without invoking a transition metal in the nitrate sensor. Both in hemocyanin and in the phosphatase, conformational changes are associated with anion binding distant from the binding site of nitrate. Thus, nitrate binding to NarX could effect the changes postulated in the above model.

IHF is the third element that is required for activating the *nar* operon (660). It is bound between NarL heptamers (888). Bending of the DNA around the IHF protein is assumed to bring NarL in contact with FNR and the polymerase (734, 960). The crystal structure of IHF bound to a target DNA has been solved recently and shows how the DNA is bent by the protein (680).

RpoN, a Mixed Situation

A switch in the transcriptional pattern, as is required for the anaerobic and N oxide-dependent expression of the entire denitrification system, could be attributed to the interaction of the core RNA polymerase with a sigma factor. In *Bradyrhizobium japonicum*, a regulatory cascade operates (Fig. 30) such that the two-component oxygen sensor-regulator pair FixJ and FixL acts on the FNR factor $FixK_2$, which, in turn, controls the expression of an *rpoN* gene. RpoN is necessary for the expression of *fix* and *nif* genes for nitrogen fixation (492). *P. aeruginosa* harbors several sigma factors; some of them, including the *rpoN* gene which encodes σ^{54} or σ^{N}, have been chromosomally mapped (356, 700). σ^{54}, first identified as a factor required for the expression of nitrogen-regulated (*ntr*) genes, is involved in the transcription of genes for a variety of physiological functions (495, 567). σ^{54} requires for binding the consensus TGG CAC-N_5-TTGCA, with the critical GG-GC sequence located at $-24/-12$ relative to the start of transcription.

RpoN-dependent genes usually require an upstream-binding activator to initiate transcription (754). To bring the activator into contact with the polymerase, looping-out of the intervening DNA is necessary, and this can be accomplished by IHF. Most of the σ^{54}-dependent promoters have the target sequence for IHF. The IHF gene has been identified in the denitrifier *P. aeruginosa* (190), and an IHF-binding motif has been suggested for *nosZ* (171).

The sequences of *rpoN* genes from *R. eutropha* (697), *P. aeruginosa* (409), and *P. stutzeri* (320) have been determined. A notable conservation of further genes in the immediate vicinity of *rpoN* (567) is also seen in these denitrifiers. Denitrification genes show no uniform requirement for σ^{54}. Mutational inactivation of *rpoN* abolishes denitrification by *R. eutropha*, indicating that a global regulator or individual denitrification genes are dependent on this factor (697). Potential σ^{54}-binding sites are present in the promoter regions of *nosZ* of *R. eutropha* (974) and *nirU* of *Pseudomonas* sp. (941). The locations of these motifs relative to the transcriptional start site and whether they represent active binding sites of σ^{54}-regulated genes are not known. Both genes are thought to be regulated by an FNR factor, but control of FNR-regulated genes by σ^{54} would be exceptional. Most of the FNR-dependent promoters are expected to function together with σ^{70}.

The *nnrR* gene has a recognition motif for RpoN which, if active, would make nitrite reduction and NO reduction of *Rhodobacter sphaeroides* dependent on σ^{54} (833). An *rpoN* mutant of *Azospirillum brasilense* is defective in nitrate assimilation, but the effect on nitrate respiration was not reported (578).

Mutagenesis of *rpoN* in *P. aeruginosa* does not affect nitrate respiration (834). The global regulator ANR of *P. aeruginosa* is synthesized constitutively. In spite of a CC-N_{10}-GC motif in the *anr* gene, which is somewhat removed from the +1 position of transcription, *anr* expression is proposed not to be σ^{54} dependent (718). An *rpoN* mutant of *S. meliloti* is not affected in N_2O reduction (357). The possibility of two functional copies of *rpoN* has to be considered, but DNA-DNA hybridization gave no clues that a gene duplication was present in *P. aeruginosa* (233) or *P. stutzeri* (883).

We have recently mutagenized *rpoN* from *P. stutzeri*. The mutated cells lose their capability to grow on minimal medium. The inactivation of *rpoN* decreases the expression of *nirS* and *norCB*. Although transient nitrite accumulation is a characteristic of the *rpoN* mutant, the overall denitrification process is not grossly affected (320). No clear consensus motifs for σ^{54} are found in the promoters of *nosZ*, *norC*, and *nirS* of *P. stutzeri*. Either putative recognition motifs are degenerate or existing GG-GC sequences show anomalous spacing (171). The involvement of RpoN in the denitrification of *P. stutzeri* must therefore be indirect, and a dependence of the transcription of the structural genes for nitrite and NO reductases on σ^{54} is not realized.

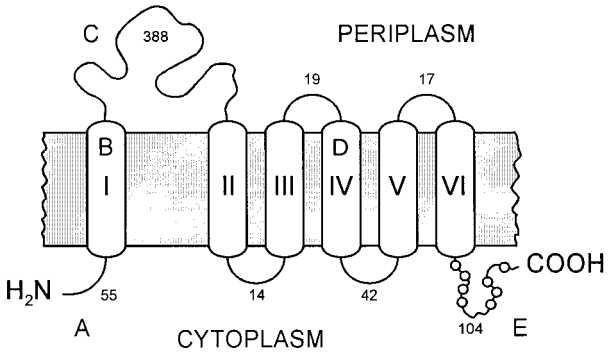

FIG. 33. Topological model of the five-domain structure of NosR from *P. stutzeri*. Domains A through E are described in the text. The circles in domain E symbolize the eight cysteine residues arranged in two clusters. Arabic numerals give the number of amino acid residues forming the periplasmic and cytoplasmic loops connecting the helices. The topology is derived from LacZ fusions in domain C (171) and the TopPredII algorithm (151).

NosR, a Membrane-Bound Regulator?

Tn*5* insertions in a 2.3-kb region upstream of *nosZ* of *P. stutzeri* result in strains with defective N_2O utilization that range in their phenotype from a complete lack of N_2O reductase synthesis to trace expression. The expression depends on the proximity of Tn*5* to *nosZ*, perhaps due to a weak internal promoter activity from the transposon. Nitrite and NO respiration are not affected in such mutants (172, 971). The mutated region comprises a single ORF designated *nosR*. The deduced product (81.9 kDa) has the architecture of a transmembrane-functioning or signaling protein, with a five-domain structure extending on either side of the cytoplasmic membrane (Fig. 33). NosR is preferentially expressed under anaerobic conditions in the presence of nitrate. Its synthesis may be under the control of an FNR-like factor, consistent with the presence of a recognition motif, although quite distant from the transcription start (171).

The N-terminal domain A of about 55 amino acids is hydrophilic and is predicted to be cytoplasmic. It is followed by the hydrophobic domain B, which provides a membrane anchor for domain C, a sizable hydrophilic region of about 400 amino acids. The periplasmic location of this domain is supported from translational PhoA fusions at Asp242 and Glu392 (171). The previous assumption of domain C as being cytoplasmic, based on the prediction of a helix-turn-helix motif, conflicts with our more recent experimental data. Transmembrane domain B is likely to be specifically required for the export of domain C, which otherwise would be cytoplasmic (843). Domain D is a five-helix bundle which is followed by the hydrophilic, cytoplasmic domain E, containing two motifs of four cysteine residues each. These motifs show a resemblance to bacterial [4Fe-4S] ferredoxins and were proposed to form metal-binding centers (172). Evidence is accumulating for regulatory roles of Fe-S proteins. Mutagenesis directed at the presumed metal-binding cysteines of NosR and deletions in the periplasmic domain are feasible experimental approaches that should help to clarify the role of NosR.

A role in electron transfer from the membrane toward the periplasmic NosZ, ascribing NosR quasi-status of a membrane-bound N_2O reductase subunit, is less likely given the topological situation of the putative metal cluster facing the cytoplasm, as well as the effect of NosR on *nosZ* transcription. *nosZ* has a monocistronic transcript, which is also suggested for *nosR* from complementation analysis (172). The *nosZ* transcript should thus not be affected by a mutation in *nosR*. However, a *nosR* mutant lacks the *nosZ* transcript, which supports a *trans*-acting function for NosR.

The membrane-bound nature and multidomain structure, including putative Fe-S centers, make the NosR protein an interesting component whose function may be accessed from homologous or related proteins. The similar *nirI* gene product (70 kDa) of *Paracoccus denitrificans* affects *nirS* expression. With a *lacZ* fusion, it was shown that the *nirS* promoter is inactive in a *nirI* mutant (184). A degenerate FNR box is located between *nirS* and *nirI*, raising the possibility of anaerobic regulation of *nirI*. Further NosR proteins are encoded by genes upstream of *nosZ* of *P. aeruginosa* (974), *Paracoccus denitrificans* (349), *S. meliloti* (357), and "*A. cycloclastes*" (556), which makes this component common to N_2O-respiring bacteria. NosR and NirI may fulfill a similar function but act on different target genes, not unlike the situation described above with FNR homologs.

The domain structure of NosR resembles that of the presumed Fe-S proteins RdxA of *Rhodobacter sphaeroides* and FixG of *Bradyrhizobium japonicum*, although in these cases with a different domain order of A-D-E-B-C (NosR lettering) (605, 657). The periplasmic and cytoplasmic domains for RdxA are supported by PhoA fusions. FixG is believed to be an oxidoreductase for Cu and to be part of a membrane-bound Cu-processing complex necessary for the biosynthesis of the cytochrome cbb_3 heme-Cu oxidase. The *fixG* gene is part of a *fixGHIS* operon, positioned immediately downstream of the oxidase genes *fixNOQP*. An enzymatic role for NosR in the maturation process of NosZ is difficult to reconcile with its effect on *nosZ* expression or that of its homolog NirI on *nirS* expression, and regulatory roles remain the preferred view.

THE ORGANISMIC SIDE

Bacterial Diversity in Denitrification

Bacteria with the capability of denitrification belong to a broad variety of groups and encompass a wide range of physiological traits. Denitrifiers are somewhat more frequent within the alpha and beta classes of the *Proteobacteria*, although there is no recognizable pattern of distribution. Denitrification is notably absent from the enterobacteria which respire nitrate to nitrite and direct the further reduction of nitrite to ammonification. Hyperthermophily (368, 881) and alkaliphily (65) are traits newly recognized among the denitrifying prokaryotes. Table 11 lists genera of denitrifying species grouped according to their principal growth mode or an otherwise dominant physiological feature (965). New descriptions of denitrifiers not mentioned elsewhere in this article are *Acidovorax delafieldii* and *Acidovorax temperans* (903), *Hyphomicrobium denitrificans* (848), and *Brachymonas denitrificans* (343).

Most denitrifiers are aerobic heterotrophic organisms that transfer redox equivalents from the oxidation of a carbon source to an N oxide under anaerobic conditions. Autotrophic denitrifiers utilize inorganic sulfur compounds, hydrogen, ammonia, or nitrite (Table 11). Recently it was found that the oxidation of Fe(II) is also coupled to a complete denitrification process: $10FeCO_3 + 2NO_3^- + 24H_2O \rightarrow 10Fe(OH)_3 + N_2 + 10HCO_3^- + 8H^+$ (797). The reaction is carried out by established denitrifiers as well as by new gram-negative isolates awaiting classification. Among the carboxidotrophic bacteria, only *Pseudomonas carboxydohydrogena* grows autotrophically under denitrifying conditions with H_2 as the electron donor and CO_2 as the carbon source (257, 568). The oxidation of CO is not coupled to denitrifying growth in the currently known carboxidotrophic bacteria. *Pseudomonas carboxydoflava* expresses the complete denitrification pathway but is sensitive to CO in the reduction of N_2O. *P. carboxydohydrogena*, *Zavarzinia* (formerly *Pseudomonas*) *compransoris*, and *Pseudomonas gazotropha* all terminate denitrification with N_2O. Denitrification and ammonification has been described for "*Pseudomonas putrefaciens*" (710), a species that belongs to the *Shewanella* branch (203).

TABLE 11. The metabolic diversity of archaeal and bacterial genera harboring denitrifying species

Archaea	Bacteria (gram-negative)
Organotrophic	Diazotrophic
Halophilic	*Aquaspirillum*
Haloarcula	*Azospirillum*
Halobacterium	*Azoarcus*
Haloferax	*Bacillus*
Hyperthermophilic	*Bradyrhizobium*
Pyrobaculum	*Pseudomonas*
	Rhodobacter
Bacteria (gram-positive)	*Rhodopseudomonas*
Organotrophic	*Sinorhizobium*
Spore forming	Thermophilic
Bacillus	*Aquifex*
Nonspore forming	*Bacillus*
Jonesia	*Thermothrix*
	Psychrophilic
Bacteria (gram-negative)	*Aquaspirillum*
Phototrophic	*Halomonas*
Rhodobacter	Halophilic
Rhodopseudomonas	*Halomonas*
Rhodoplanes	*Bacillus*
Lithotrophic	Pigment-forming
S oxidizing	*Chromobacterium*
Beggiatoa	*Flavobacterium*
Thiobacillus	*Pseudomonas*
Thioploca	Budding
H_2 oxidizing	*Blastobacter*
Ralstonia	*Hyphomicrobium*
Paracoccus	Gliding
Pseudomonas	*Cytophaga*
NO_2^- or NH_4^+ oxidizing	*Flexibacter*
Nitrobacter	Magnetotactic
Nitrosomonas	*Magnetospirillum*
Organotrophic	Pathogenic
Carboxidotrophic	*Achromobacter*
Pseudomonas	*Alcaligenes*
Zavarzinia	*Agrobacterium*
Oligocarbophilic	*Campylobacter*
Aquaspirillum	*Eikenella*
Hyphomicrobium	*Flavobacterium*
Fermentative	*Kingella*
Empedobacter	*Moraxella*
Azospirillum	*Morococcus*
Facultatively anaerobic	*Neisseria*
Alteromonas	*Ochrobactrum*
Pseudomonas	Oligella
Aerobic	*Pseudomonas*
Paracoccus	*Sphingobacterium*
Alcaligenes	*Tsukamurella*

TABLE 12. Taxonomic transfers of denitrifying bacteria and archaea

Former genus and species	Valid designation	Reference
Bacteria		
Achromobacter xylosoxidans	*Alcaligenes xylosoxidans* subsp. *xylosoxidans*	455
Alcaligenes denitrificans	*Alcaligenes xylosoxidans* subsp. *denitrificans*	455
Alcaligenes eutrophus	*Ralstonia eutropha*	927
Aquaspirillum magnetotacticum	*Magnetospirillum magnetotacticum*	730
Campylobacter cryaerophila	*Arcobacter cryaerophilus*	856
Erythrobacter strain OCh114	*Roseobacter denitrificans*	750
Paracoccus halodenitrificans	*Halomonas halodenitrificans*	207
Pseudomonas avenae	*Acidovorax avenae* subsp. *avenae*	904
Pseudomonas compransoris	*Zavarzinia compransoris*	571
Pseudomonas glumae	*Burkholderia glumae*	847
Pseudomonas perfectomarina	*Pseudomonas stutzeri* ZoBell	210
Pseudomonas pseudoflava	*Hydrogenomonas pseudoflava*	902
Pseudomonas putrefaciens	*Shewanella* sp.	203
Pseudomonas solanacearum	*Burkholderia solanacearum*	926
Rhizobium meliloti	*Sinorhizobium meliloti*	189
Rhodopseudomonas sphaeroides	*Rhodobacter sphaeroides*	379
Rhodobacter sphaeroides f. sp. *denitrificans*	*Rhodobacter sphaeroides* IL106	344
Rhodopseudomonas capsulata	*Rhodobacter capsulatus*	379
Thiobacillus versutus	*Paracoccus versutus*	442
Thiosphaera pantotropha	*Paracoccus denitrificans* GB17	524
Vibrio succinogenes	*Wolinella succinogenes*	810
Archaea		
Halobacterium denitrificans	*Haloarcula denitrificans*	825
Halobacterium marismortui	*Haloarcula marismortui*	623
Halobacterium vallismortui	*Haloarcula vallismortui*	830

Denitrification research encompasses a strong organismal aspect. Comparative investigations and evolutionary considerations require the consolidation of the systematic position of a denitrifying bacterium. A number of recent transfers are listed in Table 12. Attention has been drawn previously to the fact that "*Pseudomonas denitrificans*" has no valid taxonomic standing (965). A particular isolate of "*P. denitrificans*" has contributed much to the enzymology of denitrification. It was the source organism for the first Cu-containing nitrite reductase (393) and belongs to *Alcaligenes xylosoxidans* subsp. *xylosoxidans* (455). In the literature it is also found as *Achromobacter xylosoxidans*, *Alcaligenes xylosoxidans* NCI(M)B 11015, and *Alcaligenes denitrificans* subsp. *xylosoxidans*. An uncertain situation continues for *Alcaligenes faecalis* IAM1015 and "*A. cycloclastes*" IAM1013, since their properties distinctly deviate from those of the respective type strains (619). The classification of *Paracoccus halodenitrificans* has been reevaluated on the basis of 16S rRNA, with the result that this bacterium is inappropriately placed in the genus *Paracoccus*. It is now a member of the family of the *Halomonadaceae* (207).

Diazotrophy. Among the diazotrophic bacteria, denitrification was detected in *Azospirillum* (formerly *Spirillum*) *lipoferum* (179), *A. brasilense* Sp7 (180), and the microaerophilic *Magnetospirillum magnetotacticum* (45). *Bradyrhizobium japonicum* and a considerable number of other rhizobia are able to denitrify (618). *Azoarcus tolulyticus* is a diazotrophic toluene-degrading bacterium which exhibits complete denitrification (961); *A. evansii* degrades a variety of monoaromatic compounds under denitrifying conditions but is unable to use toluene (12). Diazotrophic denitrifiers are also found among the phototrophic bacteria.

Denitrification and diazotrophy can proceed concomitantly. The end product of the former process was shown to be diverted to nitrogen fixation (228). Among the pseudomonads, diazotrophic strains have been reported for *P. fluorescens*, *P. putida* (not a denitrifier), and *P. stutzeri* (133). *P. stutzeri* CMT.9.A fixes N_2 (479) and hybridizes with gene probes for the N oxide reductases from the ZoBell strain (unpublished data). The pseudomonadal nitrogenase has not been studied in any detail. "*P. pseudoflava*" is a denitrifying bacterium of which several diazotrophic strains have been isolated whose genes bear homology to the *nifDHK* genes of the Mo-dependent nitrogenase (402). The bacterium is now a member of the new genus *Hydrogenophaga* (902).

Phototrophy. The first isolate of a photosynthetic denitrifying bacterium, *Rhodobacter sphaeroides* f. sp. *denitrificans* IL106 (717), is a regular strain of *R. sphaeroides* because of the 16S rRNA similarity (344). In this article, it is referred to by its strain designation. IL106 synthesizes a Cu-containing nitrite reductase (720). *R. sphaeroides* 81-3 and 2.4.3 were identified as denitrifiers by the use of antisera against the denitrification enzymes from IL106 (576). From strain 2.4.3, the genes for the Cu-containing nitrite reductase, *nirK*, and its regulator, *nnrR*, were isolated (Table 2). A close relative of *R. sphaeroides* is *Rhodobacter azotoformans*, and more denitrifying members within the genus *Rhodobacter* are anticipated (341, 342). *Rhodoplanes elegans* and *Rhodoplanes roseus* (345) are newly identified phototrophic denitrifiers. The genus *Rhodopseudomonas* harbors denitrifying strains within the species *R. palustris* (658).

Denitrification can be cryptic with respect to the expression of a distinct reaction step. *Rhodobacter capsulatus* BK5 harbors a membrane-bound nitrate reductase (36) and an NO reductase (59) but is not considered a true denitrifier since it lacks nitrite reductase. However, BK5 is induced to a complete denitrification pathway by repeated subculturing with a low-nitrate supplement and "gains" a Cu-containing nitrite reductase (682). It is not clear what keeps certain denitrification genes cryptic, but it may be related to the phenomenon of loss of this property.

The genus *Roseobacter* comprises two species of ovoid or rod-shaped gram-negative bacteria that exhibit aerobic phototrophic activity and synthesize bacteriochlorophyll *a* (750, 755). *R. denitrificans* grows on high-tidal seaweeds and denitrifies nitrate to N_2O. The term "paraphotosynthetic" has been proposed to describe these aerobic, bacteriochlorophyll-containing bacteria (276). *R. denitrificans* has a cytochrome cd_1 nitrite reductase (755). The level of cytochrome cd_1 increases on irradiation with green light by a factor of 5 to 7 and is proportional to the light intensity (805). The expression of cytochrome cd_1 in this bacterium appears to be under the control of a signal transduction system responding strongest to light of 561 nm wavelength.

Aerobic denitrification. The Delft isolate GB17, which became known as "*Thiosphaera pantotropha*" (690), constituted the paradigm of an aerobically denitrifying strain. The bacterium has been generically misplaced. On the basis of identical 16S rRNA and a high phenotypic similarity, it belongs to the genus *Paracoccus* as a strain of *P. denitrificans* (524). The DNA relatedness of "*T. pantotropha*" to the type strain of *P. denitrificans* is 85%; a 70% similarity is generally accepted for the species level. The denitrification enzymes and genes studied from *P. denitrificans* and "*T. pantotropha*" show little differences; for example, only 17 of 596 amino acid positions in the nucleotide-derived cytochrome cd_1 structures are different between strains PD1222 and GB17 (185, 261). Pending further

systematic consolidation along accepted taxonomic rules (290), the genus and species "*Thiosphaera pantotropha*" have no valid standing. Hence, the bacterium is referred to by its original strain designation.

Paracoccus denitrificans GB17 is a representative of a probably much larger group displaying both denitrification and heterotrophic nitrification (128, 693). The simultaneous function of aerobic respiration, denitrification, and nitrification in this *Paracoccus* species is thought to provide a means of disposing excess reductant (693). The aspect of redox balance has been discussed in detail elsewhere both for heterotrophic nitrification and photodenitrification (69).

The work with *P. denitrificans* GB17 has reactivated and considerably extended previous findings of aerobic denitrification (691, 693). As a result, the aerobic process is now seen as a variant represented by several denitrifiers rather than the extremely rare exception. The range of O_2 concentrations in which N oxide respiration was found is broad, and the onset of N oxide utilization differs from one denitrifier to another (692, 983). Aerobic denitrification results when activation of denitrification genes occurs at a high O_2 level. Regulatory control usually operates in favor of channeling electrons toward aerobic respiration. For instance, phase shifts between O_2 respiration and denitrification establish themselves as autonomous oscillations in a culture supplied with low oxygen concentrations (813). If O_2 does not suppress N oxide utilization, aerobic denitrification manifests itself as the corespiration of the two substrates.

No fundamental aspect that would make the aerobic variant a different type of denitrification has been uncovered. The enzymes found in aerobically denitrifying bacteria are those of their anaerobic counterparts. Purified cytochrome cd_1 (585) and N_2O reductase (68) from *P. denitrificans* GB17 both have properties identical to the enzymes from two other, anaerobically denitrifying strains of *Paracoccus*. Aerobic denitrification is not a question of the O_2 sensitivity of the reductases but, rather, one of regulation at the enzyme and genetic level. The properties of oxygen- or redox-sensing factors currently being discovered and acting directly or via signaling pathways at the transcriptional level will eventually provide the regulatory framework for aerobic denitrification. Anaerobically expressed gene products can also remain active under O_2 and can result in apparent aerobic denitrification.

P. denitrificans GB17 was lately reported to have lost its aerobic denitrification activity (177, 815) or to exhibit it only at 10% of the original level (29, 689). However, aerobic denitrification is also found in several other bacteria and is not limited to the most closely studied example (29, 87, 641, 692). Denitrification of *Pseudomonas carboxydoflava* is reported to be insensitive to 20% O_2 (568). *Magnetospirillum magnetotacticum* is a denitrifier that has an obligate requirement for oxygen. This bacterium denitrifies only under microaerophilic but not anaerobic conditions and consumes oxygen while denitrifying (45). A low level of aerobic synthesis of N_2O reductase was observed with *P. stutzeri* (467).

Denitrification is nearly exclusively a facultatively anaerobic or microaerophilic trait, which underlines the close relationship of the denitrification apparatus to the aerobic electron transport chain. Anaerobically grown cells do not lose their capacity to respire oxygen. Anaerobic cells of *Paracoccus denitrificans* GB17 corespire N_2O and O_2 (57). N_2O-respiring cells of *P. stutzeri* switch immediately to aerobic respiration when pulsed with O_2 and revert to N_2O utilization once the O_2 has been consumed (979).

With perhaps one or two exceptions, there are no examples where denitrification occurs in an obligate anaerobic bacterium. *Thiomicrospira denitrificans* has been described as an obligate anaerobic denitrifier (824). A resorcinol-degrading bacterium of unknown systematic relationship is another example of an obligate anaerobic denitrifier (294). Denitrifying strains related taxonomically to the genera *Azoarcus* and *Thauera* have the unusual property of utilizing ethylbenzene and propylbenzene exclusively under denitrifying, anaerobic conditions but not under oxic conditions (663).

Denitrifying nitrifiers. The nitrifying bacteria seemed a priori to be excluded from being considered denitrifiers because of their aerobic way of life and their indispensable function in the oxidative branch of the N cycle. This is no longer the case. Using ^{15}N as a tracer, Poth and Focht (654) demonstrated the production of N_2O from nitrite by *Nitrosomonas europaea*; a single isolate of *Nitrosomonas* sp. also formed N_2, suggesting the existence of N_2O reductase (653). These observations confirm and extend a number of earlier reports of denitrifying activity by *Nitrosomonas* species. Tracer studies also support a mechanism of gas production from nitrite rather than being a side product of ammonia (or hydroxylamine) oxidation (653, 654, 685). A Cu-containing nitrite reductase has been characterized from *N. europaea* (Table 5) (206). From *Nitrobacter vulgaris*, a nitrite reductase has been isolated which produced NO and 4% ammonia. Its activity is sensitive to DDC, which may indicate Cu as a prosthetic metal. Distinct properties of CuNIR from *N. europaea* are its membrane-bound nature and the presence of two subunits of 115 and 65 kDa (6).

Ammonia monooxygenase of the obligatorily lithoautotrophic nitrifier *N. europaea* catalyzes the initial activation of ammonia: $NH_3 + O_2 + 2e^- + 2H^+ \rightarrow NH_2OH + H_2O$. The monooxygenase is difficult to handle because of its instability in cell extracts. The enzyme is inhibited by acetylene, a property that has been used to radiolabel it for identification. Ammonia monooxygenase is probably a Cu protein (232). Unlike the enzyme from a chemolithotrophic source, its homolog from the heterotrophic nitrifier *Paracoccus denitrificans* PD1222 is active in cell extracts. The enzyme was recently purified and found to be a heterodimer (38 and 46 kDa) whose activity depends on Cu (586). The subunit composition corresponds to the two structural genes found for this enzyme in *N. europaea* (558). With the possibility of obtaining catalytically active ammonia monooxygenase from a denitrifier, the solution of a recalcitrant problem of isolating and characterizing a thus far missing enzyme of the N cycle has taken a significant step forward.

The product of the monooxygenase reaction is further oxidized by hydroxylamine oxidoreductase: $NH_2OH + H_2O \rightarrow NO_2^- + 5H^+ + 4e^-$. The enzyme from *N. europaea* contains seven heme C molecules and one heme P460 in each 65-kDa subunit of a homotrimeric complex (26). The crystal structure of this enzyme has been resolved. It shows an intriguing array of heme clusters, possibly because of alternative electron transfer pathways leading either to a terminal oxidase or to the reversed electron flow for pyridine nucleotide reduction (378). The enzyme from the heterotrophic nitrifier *P. denitrificans* GB17 is, in contrast to the *Nitrosomonas* enzyme, a nonheme Fe protein with a heterodimeric structure (897). Electron acceptors for this enzyme are cytochrome c_{550} and pseudoazurin.

Anaerobic ammonia oxidation and denitrification. On theoretical grounds, it has been argued that ammonia oxidation should sustain nitrate reduction (105). Organisms performing such a process are not known. However, in a fluidized-bed reactor treating effluent from a methanogenic reactor, the disappearance of nitrate at the expense of ammonia under concomitant formation of N_2 has been observed: $3NO_3^- + 5NH_4^+ \rightarrow 4N_2 + 9H_2O + 2H^+$ ($\Delta G^{\circ\prime} = -297$ kJ/mol of

TABLE 13. Denitrifying species of *Pseudomonas*

rRNA group I	Species awaiting grouping or incertae sedis[b]
P. aeruginosa	*P. azotoformans*
P. alcaligenes	*P. butanovora*
P. aureofaciens	*P. carboxydoflava*[c]
P. balearica	*P. carboxydohydrogena*[c]
P. chlororaphis	*P. gazotropha*[c]
P. fluorescens bv. B, C, F	*P. indigofera*
P. mendocina	*P. lemoignei*
P. mucidolens	*P. marginalis*
P. pseudoalcaligenes	*P. nautica*
P. stutzeri	*P. nitroreducens*
rRNA group II[a]	
P. caryophylli	
P. mallei	
P. pickettii	
P. plantarii	
P. pseudomallei	

[a] The entire homology group comprises the new genus *Burkholderia* (847, 926).
[b] The taxonomic standing of these species is discussed in references 633 and 203.
[c] Denitrification is described in reference 257.

NH_4^+). The process is termed "anammox" for anaerobic ammonium oxidation (593). The organism(s) catalyzing this reaction has not been isolated; the presumption is that it is not due to the activity of a currently known nitrifying bacterium. Dominant organisms in the anammox reactor are irregularly shaped gram-negative bacteria which possibly contain cytochromes and ether lipids. This raises the possibility of an archaeal source of this reaction (857). Antibiotics and uncouplers inhibit the process. From $^{15}NH_4^+$ and $^{14}NO_3^-$, one finds nearly quantitative amounts of $^{14\text{-}15}N_2$ (98.2%). It is assumed that N_2 is formed from equimolar amounts of nitrite and ammonia rather than nitrate (859). Nitrite is believed to be converted to hydroxylamine, which, in turn, serves as the presumed oxidant for ammonia, yielding hydrazine (858). Successive deprotonation via diimine as intermediate is believed to give dinitrogen. Diimine and hydrazine are the likely intermediates in the nitrogen fixation process. The enzymology of anammox has to be unraveled; NO and N_2O do not seem to play a role in the process.

Mineralization of monoaromatic compounds by denitrifiers. The number of new isolates of denitrifiers that mineralize a wide spectrum of aromatic compounds under anaerobic conditions is steadily growing. Substantial mechanistic advances in understanding the anaerobic degradation of benzoate have been achieved with the denitrifying species *Thauera aromatica* and *Azoarcus evansii*. The range of substrates utilized under denitrifying conditions includes toluene, xylene, phenols, cresols, phthalate, cyclohexanol, benzoate and other aromatic acids, alcohols, and aldehydes. From the broad substrate spectrum utilized and the variety of bacteria catalyzing these degradative processes, one must conclude that the role of denitrification in anaerobic mineralization is significant and complements modes of degradation of aromatic compounds under anoxic conditions found in photosynthetic, methanogenic, and sulfate-reducing bacteria (321, 328). Anaerobic toluene degradation is widespread in nature and is attributed to the activity of *Azoarcus tolulyticus* (254, 961).

Denitrifying Pseudomonads

Since denitrification cannot be studied with enterobacteria, the pseudomonads seem to be the logical choice as the alternative group. Pseudomonads contribute a sizable number of denitrifying bacteria within a single genus. They belong to the gamma subclass of the *Proteobacteria*. Pseudomonads possess no fermentative metabolism, but many of them have the ability to denitrify (Table 13). Anaerobic life at the expense of substrate-level phosphorylation is possible in certain species via arginine catabolism (311). Members of Palleroni's rRNA group I cluster taxonomically tightly and represent the authentic genus *Pseudomonas* (447, 590). The genus was named by W. Migula. Although the year 1894 is commonly cited (633), Migula's work appeared in 1895 in issue 2 of the publications from the Bacteriological Division at the Karlsruhe Technical Institute (now the University Fridericiana) of the former grand duchy of Baden.

Palleroni's rRNA homology group II comprises species that are pathogenic for humans, plants, and animals. Based on 16S rRNA, DNA homology, lipid and fatty acid composition, and phenotypic traits, these species have been transferred to the new genus *Burkholderia*, with *B. cepacia* as the type species (847, 926). The type strains of *B. mallei*, *B. pseudomallei*, *B. picketti*, and *B. solanacearum* do not produce gas from nitrate (926), but other strains have been reported to do so (633). Because of their close relatedness, *P. aureofaciens* and *P. chlororaphis* were proposed to be considered to be two strains of the same taxon, *P. chlororaphis* (417).

Among the pseudomonads, much work on denitrification has been done with two strains of *P. stutzeri*. The ZoBell strain (formerly *P. perfectomarinus* [210]) is a marine isolate; strain JM300 originates from Davis, Calif. (121). *P. stutzeri* (termed *Bacillus denitrificans* II by its discoverers R. Burri and A. Stutzer in 1895) was found to be a vigorous denitrifier that was capable of growth on nitrate, nitrite and nitrous oxide. Beijerinck and Minkman (52) suggested that of the strains used by Gayon and Dupetit (the early pioneers of denitrification), the bacterium β was identical to *Bacillus stutzeri* (now *P. stutzeri*) used in their own work.

P. stutzeri was proposed in 1983 as model organism for denitrification research (120), a choice amply justified since then. The property of *P. stutzeri* to grow on N_2O allowed for a convenient screening of mutants and opened the way for the genetic analysis (877) that was soon followed by the identification of a 30-kb denitrification gene cluster (100). *P. stutzeri* ZoBell and JM300 both show natural transformation (121, 521, 790). *P. stutzeri* is well suited for genetic manipulation. The very first examples of the structural genes for the N_2O reductase and NO reductase were isolated from strain ZoBell. Plasmids appear not to be frequent in *P. stutzeri*, since few plasmid-borne traits have been reported.

Although clinical isolates are known, there is no evidence that *P. stutzeri* is a pathogen or commensal of etiological significance (127, 359). Its natural habitat is soil, manure, mud, and stagnant water. In addition to strain ZoBell, an isolate off the Californian coast, marine isolates of *P. stutzeri* have been obtained from the Mediterranean Sea (702). Strain ZoBell is still able to multiply under 6×10^4 kPa of pressure (964). The disadvantage of *P. stutzeri* with respect to a chromosomal map and availability of markers is overcome by the advances in chromosome-mapping and genome-sequencing techniques. The advantage of *P. aeruginosa* with respect to genetic analysis is set off by its inability to grow on N_2O or by its sluggish growth at best (47, 120).

A number of taxonomic studies have been directed at *P. stutzeri* but have not yet provided a definitive picture. The most conspicuous difference of genomic characteristics is a subdivision between strains with a low G+C content (approximately 62 mol%) and those with a high G+C content (64 to 65 mol%). Strains of *P. stutzeri* cluster in seven genomic groups

(genomovars) which are distinguishable by their G+C content and have distinctive features in their 16S rRNA (60). Strain ZoBell belongs to genomovar 2 (mean G+C content, 62.0 mol%) (60, 703). Given the low correlation between genotypic and phenotypic analyses, no sufficiently uniform grouping exists yet that would justify the taxonomic regrouping of *P. stutzeri* (665, 702). The two strains of genomovar 6 were recently placed in the new denitrifying species *Pseudomonas balearica* (60). *P. stutzeri* JM300 belongs to a separate, eighth genomovar (278).

Archaea

The halobacterium from the Dead Sea, "*Halobacterium marismortui*" (redescribed as *Haloarcula marismortui* [623]) was the first archaeon shown to produce gaseous N oxides from nitrate (899). The predominant gas species produced was identified as N_2O by mass spectrometry. Nitrate reductase and nitrite reductase in cell extracts were active with MV; nitrite reductase was also active with ferredoxin from this organism.

Other halobacteria that produce gas from nitrate or nitrite are *Haloarcula* (formerly *Halobacterium*) *denitrificans* (827), *Haloarcula hispanica* (428), *Haloarcula* (formerly *Halobacterium*) *vallismortui* (830), *Haloarcula japonica* (806), and *Halobacterium mediterranei* (694). Nitrate-reducing and denitrifying activities are induced under anaerobic growth conditions only in the presence of nitrate. A complete denitrification pathway with N_2 formation from nitrate occurs in *H. denitrificans* (827). N_2O reduction is inhibited by acetylene, a property identical to the bacterial N_2O reductase. In cases where gas production was shown qualitatively in Durham tubes only, both N_2 and N_2O must be considered to be products of denitrification, with a complete or truncated pathway, respectively.

An important question is to which extent the archaeal enzymes follow the patterns of the bacterial enzymes. Nitrate reductases have been studied to a certain extent, and the first example of a nitrite reductase has been isolated. The properties of these enzymes are reminiscent of the bacterial enzymes with certain modifications and, pending more detailed studies, do not appear to represent novel proteins.

The nitrate reductase of *H. denitrificans* is membrane bound but easily solubilized by diluting membranes in buffer (347). The enzyme consists of two subunits of 116 and 60 kDa, similar to the bacterial nitrate reductase; it is active and stable in the absence of salt. The presence of Mo is suggested since tungstate represses nitrate reductase synthesis. The archaeal enzyme reduces chlorate; the electron donor is MV. An equally easily extractable, salt-independent nitrate reductase with a temperature optimum around 80°C is found in *Haloferax volcani* (77). The lack of a salt requirement has been related to an bacterial origin of the archaeal nitrate reductase (347). A soluble but salt-requiring nitrate reductase was reported from *H. mediterranei* (11). In 3.2 M NaCl, the enzyme has an activity maximum at 89°C. A thermostable nitrate reductase has so far not been described for the bacteria but may exist in the hyperthermophilic *Aquifex pyrophilus*, which exhibits complete denitrification (368).

The nitrite reductase from *H. denitrificans* is a dimeric Cu-containing protein (subunit mass, 64 kDa); a certain percentage of nitrite-reducing activity is also membrane bound but is not presumed to represent a distinct enzyme (380). The antiserum against CuNIR from "*A. cycloclastes*" does not recognize the enzyme of *H. denitrificans* (158), suggesting that the differing subunit composition with respect to the bacterial enzyme may represent a true structural difference. Archaeal nitrite reductase is active in the presence of 4M NaCl and almost inactive in its absence. Absorption maxima are at 462, 594, and 682 nm, similar to those of the reductases of "*A. cycloclastes*" or *Alcaligenes faecalis* (Fig. 11). The electron donor is not known. The archaeal halocyanin, a blue type 1 Cu protein, which is also found in the denitrifying genus *Halobacterium* (724), is a potential candidate. The existence of cytochrome cd_1 in an archaeon has not been described.

For some time, it seemed that denitrification of the archaea would be restricted to the halobacteria (533) until the hyperthermophilic archaeon *Pyrobaculum aerophilum* was found to denitrify nitrate to N_2O as the major product (881). A genome project is under way for this bacterium which is likely to become the first archaeal denitrifier providing the blueprint for its denitrification apparatus. It will be intriguing to see how much of this can be recognized and functionally assigned from the current genetic analysis of the bacterial system. The hyperthermophilic, strictly anaerobic *Ferroglobus placidus* respires nitrate but not nitrite. Nonetheless, when supplied with nitrite, the organism produces a substantial amount of N_2O, hinting at the likely presence of further denitrification enzymes (885). Methanogens have not been described to denitrify, although methanogenic sludge has a high denitrification potential and contains numerous denitrifiers (659). It is unknown whether archaea are among them. Ammonification also has not been reported for the archaea.

Hyperthermophiles represent the deepest-branching and shortest lineages in both the archaea and the bacteria and are considered to be placed at the beginning of the evolution of both domains (436, 789, 915). The existence of denitrification in the hyperthermophilic branches can be viewed as an indication of an early origin and occurrence of the process before branching of the archaeal and bacterial domains. The presence of similar enzymes, as far as can be inferred from the available evidence, support this view.

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